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# SOCIETY OF AMERICAN BACTERIOLOGISTS

## ABSTRACTS OF PROCEEDINGS

THE FORTY-SEVENTH GENERAL MEETING, PHILADELPHIA, PENNSYLVANIA, MAY  
13 TO 16, 1947

G1. *The Differentiation of Paracolon Bacilli.* ISABELLE G. SCHAUB, Johns Hopkins University School of Medicine, Department of Bacteriology, Baltimore, Md.

A simple method for differentiating the so-called paracolon bacilli is based on inhibition of the growth of one group of these organisms on certain media. At the present time Difco SS agar has been found to give most satisfactory results, but the possibility of developing a suitable medium of better known composition is being investigated. On SS agar, growth of the paracolon bacilli, regarded as aberrant coliforms, is inhibited, as is that of the typical coliform bacilli. Certain other paracolon bacilli, as well as all species of *Salmonella*, *Eberthella*, *Proteus*, *Pseudomonas*, *Serratia*, and almost all *Shigella*, grow well. Inoculation with undiluted cultures gives unreliable results, but if a diluted culture is used consistently, satisfactory reproducible results are obtained. With this method all the paracolon bacilli studied were found to fall into one of four groups: (A) Growth inhibited on SS agar. (1) Aberrant coliform bacilli, including paracolon *Escherichia*, paracolon *Aerobacter*, and paracolon intermediates of the microaerogenic, anaerogenic, papillae-forming, and non-lactose-fermenting types. (B) Growth not inhibited on SS agar. (1) Hydrogen-sulfide-producing paracolon bacilli; (2) anaerogenic paracolon bacilli, Stuart's type 29911; (3) a well-defined group whose outstanding differential characteristic is the utilization of sodium malonate. Type I includes the majority of paracolon bacilli from human sources, and this method allows rapid differentiation of these organisms from *Salmonella*, *Proteus*, and other genera. Types II, III, and IV can easily be identified by other cultural characteristics.

G2. *Physiological Studies on Actinomyces griseus.* E. L. DULANEY, A. B. HODGES, AND D. PERLMAN, Merck & Co., Inc., Research and Development Department, Rahway, N. J.

The chemical changes occurring during the fermentation of the Waksman medium (1 per cent glucose, 0.5 per cent meat extract, 0.5 per cent peptone, 0.5 per cent sodium chloride) by a strain of *Actinomyces griseus* when grown in shaken Erlenmeyer flasks indicated that there are generally two phases of metabolic activity. During the growth phase the mycelium is produced accompanied by a reduction in the soluble constituents of the medium (nitrogen, carbon, phosphorus), fermentation of the available carbohydrate, production and utilization of lactic acid, a high oxygen demand ( $Q_{O_2}$  may reach 150), and little production of streptomycin. During the autolytic phase the mycelium weight decreases markedly, inorganic phosphorus and soluble nitrogen (total

nitrogen as well as ammonia nitrogen) are released into the medium, the oxygen demand drops to zero, and considerable quantities of streptomycin are produced. The pH rises gradually throughout the fermentation. Increasing the carbohydrate or phosphate content of the medium has little effect on the general changes, although it may lengthen the growth phase. Comparison of eight strains derived by different methods from this culture indicates that no significant differences can be found in the gross physiological changes, although the maximum streptomycin production ranged from 0 to 120 per cent of the parent organism. Carbon dioxide seems to be the main metabolic product of the carbohydrate under these conditions; some lactic acid is produced.

G3. *A Serological Study of Proteus mirabilis*. MARY G. WEST, M. SCHERAGO, AND R. H. WEAVER, University of Kentucky, Department of Bacteriology, Lexington, Ky.

A study has been made of eight stock strains and ten fresh isolations of *Proteus mirabilis* in order to compare the composition of their flagellar antigens and to determine whether there is any relationship of their flagellar antigens to the *Salmonella* genus and whether the phenomenon of phase variation as described for *Salmonella* also occurs in *P. mirabilis* strains. All of the strains employed possessed the typical morphological, tinctorial, cultural, and biochemical characteristics for this organism except one strain which failed to liquefy gelatin. By means of cross-agglutination reactions in ten antisera and by means of agglutinin absorption tests, the 18 strains could be placed in six flagellar groups of seven, two, one, six, one, and one strains, respectively. When the 18 strains were tested by the tube agglutination method with antisera against all known *Salmonella* flagellar types in dilutions of 1 to 100, no agglutination occurred to indicate any relationship between *P. mirabilis* strains and *Salmonella*. Attempts to demonstrate phase variation in the 18 *P. mirabilis* strains by a modification of the Gard technique proved unsuccessful.

G4. *Further Studies on Bacterial Variation and Selective Environments*. WERNER BRAUN, University of California, Department of Veterinary Science, Berkeley, Calif.

Previously presented data proved the existence of a heat-stable, filterable factor in normal serum or plasma which selectively suppressed the establishment of non-S types of *Brucella abortus*. This factor has now been found in the  $\gamma$ -globulin fraction obtained from normal bovine and human plasma. The activity of this factor is influenced by the composition of the medium in which growth takes place as well as by the status of the animal from which the serum is obtained. Thus, the selective action is greatly altered if, instead of normal serum or  $\gamma$ -globulin, small amounts of serum from *Brucella*-infected or vaccinated animals are added to *in vitro* cultures. An alteration of the selective effect of normal serum also appears to take place during pregnancy. The possible significance of these observations for diagnostic and therapeutic procedures will



be pointed out. Additional studies have been made on the occurrence of independent changes of characteristics during variation in *Brucella abortus*. An interpretation of causative mechanisms leading to the production of linked and nonlinked changes in bacterial variation will be presented.

G5. *Isolation of Pleuropneumonia-like Organisms from Cultures of Hemophilus influenzae*. LOUIS DIENES, Massachusetts General Hospital, Department of Pathology and Bacteriology, Boston, Mass.

Cultures of *Hemophilus influenzae* show occasionally a pleomorphism similar to that observed in *Streptobacillus moniliformis* and *Bacteroides*. The bacilli swell into large round bodies which reproduce either the usual bacilli or grow into tiny colonies similar to those of the pleuropneumonia group. The fast growth of bacilli has prevented in the past the full development of the pleuropneumonia-like colonies and their isolation in pure culture. Penicillin inhibits the growth of the bacilli. It does not influence the pleuropneumonia-like colonies and it was possible by using penicillin to isolate these from one strain of *Hemophilus influenzae*. This culture continued to grow for several months. In several other strains of *Hemophilus influenzae*, including two type B strains, the colonies of pleuropneumonia-like organisms developed to full size, but their further cultivation was not successful. Colonies of pleuropneumonia-like organisms usually develop in abundance on plates inoculated with sputum containing *Hemophilus influenzae* if bacterial growth is inhibited by penicillin. *Hemophilus influenzae* is the fourth species of gram-negative bacilli from the cultures of which pleuropneumonia-like organisms have been isolated.

G6. *The Role of Halogens in the Gram Stain*. ERNEST HARTMAN, Kirksville College of Osteopathy and Surgery, Department of Bacteriology and Public Health, Kirksville, Mo.

In the course of other work, observations were made which suggested that the iodine might effectively combine with the bacterial cell before staining and that other halogens might be substituted for iodine in the gram stain. Parallel rows of various gram-positive and gram-negative organisms were used on the same slide. Staining, destaining, and counterstaining were identical for each slide. The usual Lugol fixing was used on one row and the other row received the experimental fixative. Lugol, very strong KI-I, KI alone,  $\text{HgCl}_2$ , and NaF gave differentiation when used either before or after the violet stain. These materials caused a precipitate when mixed directly with the violet stain. The time and concentration of a given halogen (probably ionized halogen) seemed to determine within limits the amount of destaining a given gram-positive bacteria would endure. *Sarcina* sp. vigorously treated and destained in acetone as much as 4 minutes showed an outer destained area. The gram stain seemed to depend on crystal violet, halogen, and cell material, without precedence of stain or halogen, to make the relatively insoluble stain lake rather than on a linear arrangement with cell material to which the stain attached and was bound by iodine.

G7. *Induced Mutations in Bacteria.* ORVILLE WYSS AND WILSON S. STONE, The University of Texas, Department of Bacteriology, Austin, Texas.

Mutations in bacteria were induced by growth in substrates which had been exposed to radiations before the bacteria were inoculated therein. Quantitative aspects of the mutation phenomenon observed with the various substrates give some indications as to the chemical nature of the substance involved in genetic control. The mutants discussed are those showing drug resistance. A possibility of directing the mutating effect was observed.

G8. *Serological Relationship Between Salmonellae and Non-Mannitol-fermenting Shigellae.* VIOLA MAE YOUNG, Mount Sinai Medical Research Foundation, Chicago 8, Ill.

*Shigella dysenteriae*, *Shigella ambigua*, *Shigella* sp. Sachs Q 771, 902, and 1167 were not agglutinated by any of 37 *Salmonella* "O" sera. Sera prepared against these shigellae did not agglutinate any of the 37 *Salmonella* strains. *Shigella* sp. Sachs Q 454 and 1030 were agglutinated by dilutions equivalent to 20 per cent of the final titer against sera of the homologous organisms of *Salmonella minnesota* and *Salmonella gaminara*. Absorption with *Shigella* sp. Sachs Q 454 and 1030 reduced by 5 to 15 per cent the titer of these sera against the homologous organisms. *Shigella* sp. Sachs Q 454 did not absorb agglutinins for *Shigella* sp. Sachs 1030 and vice versa. Agglutination and absorption experiments using sera prepared against *Shigella* sp. Sachs Q 454 and 1030 and "O" antigens of 37 *Salmonella* strains gave similar results. It was concluded, therefore, that the weak partial antigen common to *Shigella* sp. Sachs Q 454 and *Salmonella minnesota* and *Salmonella gaminara* is different from that shared by *Shigella* sp. Sachs Q 1030 and *Salmonella minnesota* and *Salmonella gaminara*.

G9. *Growth of Bacterial and Fungal Colonies as Seen with the Phase Microscope* OSCAR W. RICHARDS, American Optical Co., Scientific Instrument Division, Buffalo 15, N. Y.

Unstained, living bacteria growing in normal media may be seen and measured, either in bright or dark contrast, with the phase microscope. The method has been extended to cultures in petri dishes and mold growth tubes. The growth of mold mycelium and of smooth and rough colonies will be illustrated.

G10. *Demonstration, with the Electron Microscope, of a Nucleus in Bacillus mycoides Grown in a Nitrogen-free Medium.* GEORGES KNAYSI AND RICHARD F. BAKER, Laboratory of Bacteriology, Cornell University, Ithaca, N. Y.; RCA Laboratories, Princeton, N. J.; Department of Bacteriology, University of Pennsylvania, Philadelphia, Pa.

When the endospores of *Bacillus mycoides*, C<sub>2</sub>, are washed with sterile, distilled water and heavily inoculated to a nitrogen-free medium, many germinate and gradually use their supply of ribonucleic acid. The germ cells and subsequent generations of vegetative cells are transparent to electrons even at 50 or 30 kv. and show two types of opaque bodies. Bodies of the first type are relatively large

the forespore; they do not seem to divide by simple constriction; these are the nuclei of the cell. The bodies of the second type are smaller and very thin; they consist of beaded threads and granules located in the cytoplasmic membrane; they do not, visibly, take part in the formation of the endospore; there is evidence that they are endowed with synthetic power and that they are involved in the formation of cross plates at the time of cell division.

*G11. A Study, with the High-Voltage Electron Microscope, of the Endospore and Life Cycle of Bacillus mycoides.* JAMES HILLIER, R. F. BAKER, AND GEORGES KNAYSI, Department of Bacteriology, University of Pennsylvania, Philadelphia, Pa.; RCA Laboratories, Princeton, N. J.; Laboratory of Bacteriology, Cornell University, Ithaca, N. Y.

The structure of the endospore of *Bacillus mycoides*, C<sub>2</sub>, and its development from spore to spore were studied with the a high-voltage, electron microscope. The endospores, in their mother culture, vary considerably in appearance and, on that basis, may be classified in three groups. The spore protoplasm appears homogeneous and is surrounded by two coats. Of the two coats, the inner one is the more rigid and both are shed upon germination. There is no evidence for the pre-existence, in the resting spore, of a third coat which becomes the wall of the germ cell. The spore germinates usually by unilateral swelling, breaking of the rigid, inner coat, and subsequent tearing of the outer, highly elastic one; rarely, the double coat is completely severed into two halves which remain as caps around the ends of the germ cell. The germ cell does not bear flagella and its protoplasm appears homogeneous. The lipoprotein inclusions which appear in the prespore stage were observed. The forespore appears homogeneous. The sporangial cytoplasm in the neighborhood of the spore is not more opaque to the electrons than that present in the "sterile" portion of the sporangium.

*G12. Studies on the Delay in the Bacteriostatic Action of Sulfanilamide on Escherichia coli.* ANNE STEWART YOUNG, Northwestern University Medical School, Department of Bacteriology, Chicago, Ill.

The bacteriostatic action of sulfanilamide on *Escherichia coli* was investigated from the standpoint of the influence of the age of the bacterial culture on the reported delay that occurs before the bacteriostatic action is manifest. The rate and amount of growth of *E. coli* in a synthetic and a nutrient medium was accurately determined by means of turbidity readings and plate counts. In this manner it was possible to determine accurately the effect of sulfanilamide on cultures of any physiological or chronological age. It was found, under controlled conditions, that sulfanilamide, when added to both types of media together with an inoculum of *E. coli* consisting of old cells, produced prompt inhibition of growth. However, a delay in the bacteriostatic action of sulfanilamide occurred in both media only when it was added to cultures of *E. coli* just before or during the logarithmic phase of growth. Changes in the hydrogen ion concentration, or in the medium during growth—changes in the number of bacteria, sulfanilamide concentration, strain of *E. coli*, or culture type (rough or smooth)—did not in-

- G13. *The Kinetics of the Bactericidal Action of Penicillin and the Therapeutic Significance of the Blood Penicillin Level.* HARRY EAGLE, Laboratory of Experimental Therapeutics of the U. S. Public Health Service and The Johns Hopkins School of Hygiene, Baltimore, Md.

The rates at which streptococci, pneumococci, staphylococci, and spirochetes are killed by penicillin *in vitro* vary strikingly within a narrow range of penicillin concentrations. Thus, in the case of the C-203 strain of *Streptococcus pyogenes*, a concentration of 0.006 micrograms per ml had a definite, if slow, bactericidal action; while a maximal rate of killing was attained at 0.064 micrograms per ml. Beyond that level even an 8,000-fold increase in the concentration of penicillin, up to 512 micrograms per ml, had no significant effect on the rate at which the organisms were killed *in vitro*. Qualitatively similar results have been obtained with pneumococcus type I and the cultured Reiter strain of so-called *Spirochaeta pallida*. These two values, the minimum concentration at which the organisms are killed and the concentration at which the rate of death is maximal, apparently comprise the therapeutically useful range of penicillin concentrations. Higher levels at the focus of infection represent largely waste penicillin, and lower levels have little if any therapeutic effect. If the level is allowed to fall too low, the organisms may multiply sufficiently in the interval between injections to affect the outcome. The rate at which the organisms multiply *in vivo* is of obvious significance in this connection. Too long an interval between injections proved a much more serious factor with, for instance, pneumococcal infections of white mice than in experimental rabbit syphilis. The effect of the method of administration on the therapeutic efficacy of penicillin in experimental infection is quantitatively consistent with the mechanism of penicillin action outlined above.

- G14. *Studies on the Adsorption of Streptomycin and Its Antagonism by Salt.* SAM BERKMAN, RICHARD J. HENRY, RILEY D. HOUSEWRIGHT, AND JANE E. HENRY, Camp Detrick, Frederick, Md.

The interaction between streptomycin and a number of substances, and the effect of salt on this interaction, were studied. These substances included desoxyribonucleic acid, ribonucleic acid, thymonucleoprotein, albumin, globulin, fibrinogen, other serum fractions, and cellophane. The techniques used in these studies included ultraviolet and infrared absorption spectroscopy, dialysis, electrophoresis, and bioassay. Absorption of streptomycin onto a number of these substrates is reversed by salt. In certain, but not all, instances absorption apparently results in the loss of streptomycin activity as measured by disc assay.

- G15. *The Relation of Certain Structural Components of Streptomycin to Its Mode of Action.* CLARKE T. GRAY AND JORGEN M. BIRKELAND, Ohio State University, Department of Bacteriology, Columbus 10, Ohio.

The streptosonic lactone fragment appears to be the critical portion of the streptomycin molecule. Streptidine and N-methyl-L glucosamine as well as i-

inositol and guanidine do not inhibit *Escherichia coli*, nor do they interfere with the action of streptomycin on *E. coli*. Since hydroxylamine and cysteine form addition compounds on the free carbonyl group of the streptosonic lactone fragment, and since this leads to inactivation, the question is raised as to the importance of the carbonyl group. Dihydrostreptomycin possesses essentially the same activity as does streptomycin, but it is not inactivated by hydroxylamine or cysteine. Furthermore, it was found that strains of *E. coli* resistant to streptomycin are equally resistant to dihydrostreptomycin. This indicates that the carbonyl group is not necessary for the action of streptomycin. It was also found that the interference with the reduction of methylene blue by bacterial dehydrogenases in Thunberg experiments may be due to a competition with the redox dye for hydrogen. Since this type of interference by streptomycin occurs when resistant as well as susceptible cells are used, and since it does not occur with dihydrostreptomycin, it is suggested that it is a function of the carbonyl group and that it is not important in the fundamental mode of action of streptomycin.

*G16. Mode of Action of Dihydrostreptomycin.* JOHN HAYS BAILEY AND CHESTER J. CAVALLITO, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Bartz *et al.* reported that the reduced form of streptomycin, dihydrostreptomycin, shows an antibacterial activity which differs quantitatively from that of the natural antibiotic, and that the dihydrostreptomycin is not inactivated by cysteine as is streptomycin. We have found that when bacteria which are susceptible to both streptomycin and dihydrostreptomycin are grown in media containing either antibiotic in the presence of cysteine, antibacterial activity is not shown. It is suggested that the organisms oxidize the dihydrostreptomycin to streptomycin, which is then inactivated by the cysteine of the test medium. It appears that the antibacterial activity shown by dihydrostreptomycin results from its being oxidized to streptomycin by the bacteria.

*G17. A Contribution to Our Knowledge of the Mode of Action of Streptomycin.*

SAMUEL R. GREEN, New Jersey Agricultural Experiment Station, Department of Microbiology, New Brunswick, N. J.

This paper is concerned with the effect of streptomycin upon the growth and metabolism of *Escherichia coli*. The addition of 1 per cent pyruvate or fumarate salt to nutrient broth supported the growth of this organism in the presence of 10 micrograms of streptomycin per milliliter. When the salt concentration was increased to 3 per cent, growth took place in the presence of 150  $\mu\text{g}$  per ml. The streptomycin was not destroyed. Salts of succinic, formic, malic, and maleic acids also protected the organism against the antibiotic action of streptomycin. Lactose, as well as lactic, acetic, and propionic acid, glycerol, and glycerophosphate had no antagonizing effect upon streptomycin in concentrations of 10  $\mu\text{g}$  per ml. The ability of an organism to grow in the presence of 10  $\mu\text{g}$  per ml streptomycin and a four-carbon acid did not render it resistant to streptomycin.

G18. *On the Mechanism of the Development of Streptomycin Resistance.* C. PHILIP MILLER AND MARJORIE BOHNHOFF, University of Chicago, Department of Medicine, Chicago, Ill.

In the course of experiments designed to explain the rapidity with which susceptible microorganisms can develop resistance to streptomycin, the following observations were made: Equivalent inocula of meningococci were seeded to a series of plates containing graded concentrations of streptomycin. The plates were carefully examined after 1, 2, and 3 days' incubation. Concentrations of 20 to 40 micrograms per ml sufficed to prevent the growth of normal colonies. The plates in this range were sterile except for an occasional colony (1 or 2 per plate) of unusual appearance—large, moist, tinged with yellow. These colonies could all be readily subcultured to media containing high concentrations of streptomycin or to streptomycin-free media. They were virulent for mice. On concentrations of streptomycin ranging from 100 to 1,000 micrograms per ml, considerable numbers of another type of unusual colony developed. All on any one plate were identical in appearance but they differed from plate to plate, becoming larger, more moist and yellow with increasing concentrations of streptomycin. Their common characteristic was their inability to grow on streptomycin-free media. They were able to grow, however, on high concentrations of streptomycin. These findings indicate (a) that any large population of meningococci contains an occasional streptomycin-resistant individual and (b) that streptomycin in concentrations exceeding the optimal bactericidal concentration induces the development of additional variants which are resistant.

G19. *Synergism and Inhibition of Drug Resistance.* MORTON KLEIN AND LEONARD J. KIMMELMAN, University of Pennsylvania, Department of Bacteriology, Philadelphia, Pa.

Penicillin, sulfadiazine, and streptomycin were tested for their inhibitory action *in vitro*, both singly and in combination, against *Staphylococcus aureus*. After 48 hours' growth in partially inhibitory concentrations of each drug the surviving bacteria, when tested against the same drug, always showed a marked increase in streptomycin resistance, a moderate increase in penicillin resistance, and little or no increase in sulfadiazine resistance. The addition of sulfadiazine to streptomycin, or penicillin to streptomycin, resulted in a greater inhibitory activity than that possessed by either drug alone. This was not a simple additive effect since sulfadiazine in concentrations less inhibitory than penicillin was the more effective synergist when combined with streptomycin. Bacteria surviving the sulfadiazine-streptomycin combination showed a lesser increase in streptomycin resistance than those organisms surviving the penicillin-streptomycin combination, which indicated a correlation between the degree of synergism and the inhibition of resistant variants. A combination of low concentrations of the 3 drugs effectively inhibited multiplication and the bacteria surviving the 48-hour test period never showed any increase in resistance to any of the three agents. A general hypothesis for the inhibition of the development of drug resistance may be suggested; resistance develops by the selection and subsequent multiplication of variants resistant to a given concentration of drug. Any additional agent that

in itself prevents the multiplication of bacteria, whether it be added drug, antibodies, or phagocytes, will interfere with the rapid multiplication of the variants surviving the action of a single drug and thus modify the rate at which resistance develops.

G20. *The Effect of Sulfathiazole on the in Vitro Synthesis of Certain Vitamins by Escherichia coli.* A. KATHRINE MILLER, PAULINE BRUNO, AND ROSALIND M. BERGLUND, Sharp & Dohme, Inc., Department of Bacteriology, Glenolden, Pa.

The synthesis of folic acid, pantothenic acid, nicotinic acid, and biotin by a sulfonamide-resistant strain of *Escherichia coli* and its sulfonamide-sensitive parent strain was studied. The cultures were grown in a salts-glucose-asparagine medium both in the presence and absence of sulfathiazole. After the turbidities of the cultures had been adjusted to comparable levels, assays were performed using *Lactobacillus arabinosus* as the test organism for all of the vitamins except folic acid, which was measured both by *Streptococcus faecalis* R and by *Lactobacillus casei*. These assays showed that the synthesis of nicotinic acid by either the sensitive or the resistant strain of *E. coli* was not diminished by the presence of partially inhibitory concentrations of the sulfonamide. However, the synthesis of the folic acids, biotin, and pantothenic acid was markedly decreased when the sensitive strain was grown in medium containing sulfathiazole. When the sulfonamide-resistant culture is grown under similar conditions, the production of the folic acids is moderately inhibited, the synthesis of biotin is only slightly depressed, and pantothenic acid is produced in equal or greater amounts than in the control cultures. The significance of these results in relation to the mechanism of sulfonamide action is discussed.

G21. *Studies on the Mechanism of Action of Furacin or 5-Nitro-2-Furaldehyde Semicarbazone.* MORRIS N. GREEN, University of Pennsylvania, Department of Bacteriology, Philadelphia, Pa.

No change in resistance to furacin occurred when the resistance of several gram-positive and gram-negative bacteria was increased with respect to sulfathiazole, streptomycin, or penicillin. The antagonistic effect of vitamins and amino acids on the inhibitory action of furacin on *Escherichia coli* was also studied. Of all the members of the vitamin B complex tested, thiamine, calcium pantothenate, nicotinamide, and pyridoxine antagonized furacin. Similarly out of a total of 22 amino acids, the following were effective in the order listed: *l*(+) arginine, *dl*-isoleucine, *l*(+)glutamic acid, *l*(+)lysine, and *dl*-phenylalanine; *dl*-norleucine, however, inhibited growth. The significance of these observations regarding the mechanism of furacin will be discussed.

G22. *On the Mode of Action of Streptomycin.* JANE HENRY, RICHARD J. HENRY, RILEY D. HOUSEWRIGHT, AND SAM BERKMAN, Camp Detrick, Frederick, Md.

These studies were conducted on resting cell suspensions of streptomycin-susceptible and streptomycin-resistant strains of *Staphylococcus aureus*, *Bacillus*

*cereus*, and *Shigella dysenteriae* (Sonne). The aerobic oxidation of glycerol and lactate by the streptomycin-susceptible strains of all three species was inhibited by streptomycin. In addition, the aerobic oxidation of glucose, pyruvate, acetate, succinate, and ethanol by *B. cereus* was inhibited by the antibiotic. The anaerobic glycolysis of glucose and pyruvate by *B. cereus* was inhibited by streptomycin. Inhibition was observed in all cases with from one to ten units of streptomycin per ml. Very slight or no inhibition of any of the above substrates was observed with 100 units of streptomycin when streptomycin-resistant strains were used. In correlation with the antagonism by salt of streptomycin inhibition of growth, salt was found to antagonize inhibition of these metabolic functions.

G23. *A Protest Against the Misuse of the Generic Name Corynebacterium.* HAROLD J. CONN, New York Agricultural Experiment Station, Division of Food Science and Technology, Geneva, N. Y.

There has been a recent tendency to include a greater and greater variety of organisms in *Corynebacterium*, until one can almost say that, if all these forms are included, there is no reason for excluding any gram-positive nonsporeforming rods, except the lactobacilli. It was first broadened by including the "diphtheroids" of animal origin, forms which show less morphological variation than the type species, *Corynebacterium diphtheriae*. To the other extreme, Jensen has added several species of soil forms, which show even greater morphological variation than the type (branching forms, etc.) and are distinctly different physiologically from typical diphtheroids. Among the species thus added by Jensen was the plant pathogen, previously placed in *Phytomonas*, which he renamed *Corynebacterium michiganense*. This has led to a broadening of the genus in still a different direction. Dowson included two more gram-positive plant pathogens, including an organism with a single polar flagellum, and Starr has more recently added another polarly flagellated plant pathogen. This produces such a miscellaneous group that it cannot be considered a single genus. Certainly the monoflagellate plant pathogens do not belong in a genus that is primarily nonmotile (although a few doubtful forms with peritrichic flagellation have been included). Furthermore, the so-called "corynebacteria" of soil origin differ to such an extent physiologically, morphologically, and in their staining reactions that they, too, seem to belong in a separate, although perhaps closely related, genus.

G24. *A Taxonomic Study of the Mesophilic Achromobacter.* J. M. RUSH, Clemson Agricultural College, Department of Botany and Bacteriology, Clemson, S. C.

Despite the fact that genus *Achromobacter* has existed for nearly a quarter of a century, and over 100 species have been described and assigned at least tentatively to the genus, no attempt has been made to isolate and study the group. An effort has been made to bring together all of the known or suspected isolations of the members of this genus from stock and private collections. Over 400 isolations have been subjected to an extensive investigation including cultural, mor-



phological, physiological, and serological studies. The resulting data indicate that the genus is composed of a serologically heterogeneous group of species. They are, however, distinct from other groups with which they have many other characteristics in common.

The type species has been isolated, though this strain reduces nitrate and is active on litmus milk, whereas Bergey records Eisenberg's organism as negative for both of these characteristics. However, no literature is cited which confirms or denies these characteristics. This strain is positive for all of the characteristics listed in Eisenberg's description. A key has been developed for the classification of the mesophilic strains of the genus using motility, gelatin liquefaction, nitrate reduction, fermentation of glucose, and litmus milk reactions as criteria for the identification of the species retained within the genus.

*G25. A Serological Study of a Group of Citrate-positive Anaerogenic Organisms.*

ELIZABETH J. COPE AND JOSEPH A. KASPER, Department of Health, Detroit, Mich.

One hundred and four strains of anaerogenic gram-negative bacilli, isolated from fecal specimens, were studied biochemically and serologically. The organisms were all citrate-positive, did not ferment lactose, and all produced acid but no gas from glucose. H, L, and O sera were produced against each of five type strains by injecting rabbits with suitable antigens. H, L, and O antigens were prepared from each of the isolated strains under study. Agglutination tests were made using each type of antigen with the appropriate type of antiserum. Twenty-two of the isolated strains were not agglutinated by any of the sera. Of the 82 strains showing specific agglutination by homologous sera, 27 also showed cross agglutination by other sera. Organisms in this group possess H, L, and O antigens. Classification of this group by serological methods may be possible.

*G26. The Use of the Enzyme Lecithinase in Grouping Some Members of the Genus Bacillus.* ARTHUR R. COLMER, West Virginia University, Department of Plant Pathology and Bacteriology, Morgantown, W. Va.

Although morphological, cultural, and physiological characteristics have been commonly employed in classifying members of the genus *Bacillus*, the production of lecithinase for the separation of these organisms has not been employed. A spoilage of some shell eggs in which the deterioration was characterized by the formation of a sour odor, a tan-colored albumen, and a hardened yolk was found to be caused by an organism identified as *Bacillus cereus*. This spoilage was due to an extracellular enzyme hydrolyzing the phosphatid lecithin of the egg yolk. This hydrolysis so modifies the colloidal state of some constituents of the yolk that it is characteristically hardened. The following species of the genus *Bacillus* did not produce lecithinase when tested by egg inoculation or when put on McClung's egg yolk medium or in van Heyningen's egg yolk emulsion: *subtilis*, *megatherium*, *pumilus*, *alvei*, *circulans*, *brevis*, *macerans*, *polymyxa*, *sphaericus*, and *mesentericus*. On the other hand, *B. cereus* and those bacteria shown by Smith to be closely allied—*B. mycoides*, *B. albolactis*, *B. prausnitzii*, and *B. anthracis*—

produced lecithinase in varying amounts. Cultures of *B. cereus* and *B. albolactis* from different sources were strong hydrolyzers of lecithin, but the cultures of *B. mycoides*, *B. prausnitzii*, and *B. anthracis* produced less of this enzyme. A variant of *B. mycoides* also possessed the enzyme.

G27. *Differentiation of Brucella abortus Strain 19 by Dye Bacteriostasis.* II. B. LEVINE AND J. B. WILSON, University of Wisconsin, Department of Agricultural Bacteriology, Madison 6, Wis.

*Brucella abortus* strain 19, a cattle-immunizing strain which does not require carbon dioxide for growth, is usually differentiated from other strains of this species by its relative lack of virulence. The greater sensitivity of strain 19 to the bacteriostatic action of dyes permits its differentiation from all other strains tested by simple cultural methods. Using standard inocula, 50 strains of *B. abortus* which do not require increased carbon dioxide tension for growth were cultured on solid medium in the presence of varying concentrations of thionine, methylene blue, and thionine blue (British Drug Houses, London). Strain 19 is more sensitive to these dyes and can be differentiated by determining a critical dye level in the medium which selectively inhibits it. Stock solutions of thionine and methylene blue became unsatisfactory for this purpose on standing, but thionine blue showed no changes in this respect over a period of 2 months, and confirmation of the work of D. H. McLeod was obtained. Strain 19 is inhibited at a dye threshold of 0.5 mg of thionine blue per liter of Difco tryptose agar. Virulent strains grow well at this level and are inhibited only at dye concentrations ranging from 0.7 to 3.0 mg per liter. All virulent strains of *B. abortus* which have been examined can be differentiated from strain 19 by this method.

G28. *Further Studies on Lactobacillus enzymothermophilus.* THEODORE C. BUCK, JR., Baltimore City Health Department, Bureau of Laboratories, Baltimore 3, Md.

In 1942 an organism isolated from bottled pasteurized milk produced a phosphatase enzyme which was not destroyed by ordinary pasteurization temperatures. Since its biochemical reactions other than phosphatase production correlated with those of *Lactobacillus thermophilus* of Ayers and Johnson (1924) and Charlton (1932), the organism was considered a member of the genus *Lactobacillus* and named *Lactobacillus enzymothermophilus*. This classification of the organism has since been questioned. Accordingly, additional evidence has been obtained which substantiates the original assignment of this organism to the genus *Lactobacillus*. Smooth and rough variants were differentiated and the biochemical reactions of each studied. The viability of the organism was again studied and its failure to produce spores was confirmed repeatedly.

G29. *Taxonomy of the Genus Lactobacillus, with Special Reference to Correlations of Differential Characteristics.* RALPH P. TITSLER, DONNA S. GEIB, AND MORRISON ROGOSA, U. S. Department of Agriculture, Agricultural Research Administration, Washington, D. C.

It is generally recognized that the literature concerning the taxonomy of *Lactobacillus* lacks agreement and completeness in many respects and that accurate identification and differentiation of species have been difficult, and frequently uncertain. An extensive study has been made of approximately 250 strains of *Lactobacillus*, comprising most species, to determine the distinctive characteristics which can be employed for classification species or types within the genus. The following characteristics were determined: morphology, colonial type, percentage of acid formed in milk, rotation of acid, limits of temperature for growth, tolerance to sodium chloride, fermentation of 24 carbohydrates, liquefaction of gelatin, production of catalase, gas, and ammonia. The findings reveal distinctive correlations among characteristics, permitting the use of a few easily and rapidly conducted tests for the identification of species or types. Contrary to conclusions drawn by some investigators, fermentative abilities can be employed advantageously in the taxonomy of *Lactobacillus*. The designation *Lactobacillus bulgaricus* has been employed by many workers for either of two distinctly different species, *Lactobacillus bulgaricus* and *Lactobacillus lactis*. Many strains designated as *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* are *Lactobacillus casei*. A simple key to the species of the genus is proposed. It is based on correlations of characteristics, chiefly on limits of temperature for growth, colonial type, production of gas, percentage of acid formed in milk, rotation of acid, and fermentation of certain carbohydrates. Strains which differ slightly from typical strains of a recognized species are designated as atypical strains or variants.

G30. *Correlation of Vitamin Requirements and Cultural and Biochemical Characteristics of the Genus Lactobacillus.* M. ROGOSA, R. P. TITSLER, AND D. S. GIBB, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington 25, D. C.

Although lactobacilli are employed in nutritional studies and assays of vitamins, no comprehensive results relating nutritional requirements to cultural and biochemical characteristics are available. In conjunction with other studies, the requirements for the B vitamins of approximately 250 strains have been determined. In a semisynthetic medium containing all known B vitamins the following strains do not grow: *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus leichmannii*, *Lactobacillus garyonii*, and a few unclassified strains.

All cultivatable strains which did grow required pantothenic acid and biotin. All strains requiring thiamine are heterofermentative. All strains which grew without extraneous riboflavin fermented either melibiose, raffinose, or inulin. This may indicate a common enzyme system essential for the cleavage of levulose or galactose from these carbohydrates. Of 47 strains which grew without folic acid, 45 ferment melibiose, or raffinose, or inulin, or arabinose, or xylose. All strains growing without folic acid grew also without pyridoxine, but the converse is not true. All gas-forming strains and also those fermenting either arabinose or xylose grew well without pyridoxine. Nearly all strains grew without choline

inositol, and *p*-aminobenzoic acid. Technical difficulties in the determination of the requirement for nicotinic acid are being studied. These results correlate well with the cultural and biochemical characteristics conventionally used in the study of lactobacilli and make available a practical method for use in taxonomy.

*G31. The Ability of Peptones to Support Surface Growth of Lactobacilli.* HARRIETTE D. VERA, Baltimore Biological Laboratory, Baltimore 18, Md.

Four peptones and yeast extract were studied for their ability to support growth of lactobacilli on the surface of agar media incubated aerobically. Peptic digest of meat, pancreatic digest of lactalbumin, pancreatic digest of casein, and papain digest of soy meal were used. The peptones and yeast extract were made up singly or in combination in 2 per cent concentration with 1.0 per cent glucose, 1.2 per cent agar, and 0.2 per cent NaCl. Fifty-nine strains of lactobacilli previously maintained in deep agar cultures or in thioglycolate broth were streaked on slants or plates. After incubation, the presence or absence of visible growth was recorded and also the size and number of colonies and the length of incubation required. Good surface growth was obtained with lactalbumin peptone agar, especially when 0.3 per cent sodium citrate was incorporated. On repeated aerobic subculture, there was a tendency toward increased rate of growth and colony size. Addition of tyrosine, alanine, cystine, and glycine had no appreciable effect. Casein digest with either lactalbumin or soy peptones made satisfactory substrates when cystine (0.05 per cent) was added. Yeast extract supported growth of about half the test organisms. The meat peptone gave the poorest results: none of 17 strains tested grew on plates incubated in air, although 5 of them showed good colony formation when plates were incubated in candle jars. It was concluded that aerobic surface cultivation of lactobacilli may be accomplished in media containing suitable peptones.

*G32. Antigen and Antibody Relationships of Oral Lactobacilli.* NED B. WILLIAMS, University of Chicago, Walter G. Zoller Memorial Dental Clinic and Department of Bacteriology and Parasitology, Chicago, Ill.

Rabbits were made hypersensitive to extracts from lactobacilli of oral origin by vaccination with heat-killed culture suspensions. Study of the sera from these sensitized animals revealed that homologous culture suspensions were agglutinated in low titer and that some degree of cross relationships existed between heterologous strains. These differences were studied further, by means of agglutination and agglutinin absorption tests, with sera having higher agglutinin titers, prepared by repeated intravenous injection of heat-killed organisms. Nine strains, selected on the basis of characteristics found in previous studies of biochemical and precipitation studies, were used for detailed study. Agglutination and agglutinin absorption tests led to the identification of four distinct antigenic components designated A, B, C, and D. Evidence was found for the presence of a fifth antigenic component. Preparation of monospecific antisera made it possible to isolate and separate components responsible for the complicating reactions previously encountered in agglutination, precipitation, and hypersensitivity tests.

G33. *Three New Species of the Genus Clostridium.* ROBB SPALDING SPRAY, The School of Medicine, West Virginia University, Bacteriology and Public Hygiene, Morgantown, W. Va.

This report describes briefly three nonpathogenic anaerobes believed to be previously unrecorded, and assigns to them suitable names. *Clostridium nau-seum*: isolated thrice from soil. It is a rather large rod with subterminal spores which swell the rods. Cultures have a foul, fecal, nauseating odor, from which the name derives. *C. microsporum*: a tiny navicular organism with a small spherical, central to excentric spore, slightly swelling the rod. It was isolated with some difficulty from a case of peritonitis. Its name derives from its tiny spherical spore. *C. gummosum*: an organism of the butyric group, isolated once from gaseous gangrene and twice from normal human feces. It shows "stormy fermentation" of milk, absence of capsule, active motility—but, above all, colonies in Difco liver veal agar are so tenacious that an inserted needle draws the colony, unbroken, through the 2 per cent agar. It is a rather large rod with large ovoid, excentric spores which distinctly swell the rods. Its name derives from its extremely gummatous character. A complete description will be presented at a later date.

G34. *Studies on the C<sub>4</sub> Dicarboxylic Acid Metabolism of Bacteria.* CHESTER I. RANDLES AND JORGEN M. BIRKELAND, Ohio State University, Department of Bacteriology, Columbus, Ohio.

The literature reveals conflicting reports on the anaerobic utilization of malate and fumarate by bacteria. Our strains of *Aerobacter aerogenes* and *Escherichia coli*, although giving abundant anaerobic growth on glucose, do not grow anaerobically on malate or fumarate. This failure is difficult to understand in view of present concepts of the metabolism of these two acids. *E. coli* grew anaerobically in 48 to 72 hours on fumarate when formate was also present. Malate and succinate failed to support growth under these conditions. The relation of these findings to the metabolism of the C<sub>4</sub> dicarboxylic acids will be discussed.

G35. *Ammonium as the Primary Nitrogen Source in the Nutrition of Brucella abortus Strain 19.* PHILIPP GERHARDT AND J. B. WILSON, University of Wisconsin, Department of Agricultural Bacteriology, Madison, Wis.

Chemically defined media for *Brucella* sp. with inorganic ammonium salts as the primary source of nitrogen have been described by several workers, but were found to be unsatisfactory for *Brucella abortus* strain 19, a relatively avirulent strain important in animal immunization work. Experimental data were obtained which contribute to the resolution of the nutritional requirements of this organism. Respiration studies showed that lactate gave much higher Q<sub>o2</sub> values than did glucose or glycerol, normally used as energy sources for *Brucella*. If lactate was employed as the energy source in a suitable mineral salts basal medium containing the required accessory growth factors, the organism was capable of growth with ammonium sulfate as the principal nitrogenous compound present. Glucose did not replace lactate in this respect, nor was it stimulatory in the presence of lactate. When used supplementary to lactate, glycerol in-

creased yields; singly, it supported but slight growth. The effect of glycerol may be primarily physical rather than nutritive. Although relatively large inocula were found to be necessary, evidence indicated that nutritive carry-over was at best but a partial explanation of the failure of small inocula to initiate growth.

G36. *Effects of Oxygen on Rate of Growth and Metabolism of Brucella.* EVELYN SANDERS AND FRANCES B. RICE, Michigan State College, Department of Bacteriology and Public Health, East Lansing, Mich.

Data have been obtained which show that there is a close relationship between constituents of the medium and atmospheric conditions for maximum growth and metabolism of *Brucella* organisms. These studies have been conducted in a basal medium containing tryptose, glucose, and thiamine hydrochloride. The cultures were agitated during the growth period. The rates of growth and metabolic changes in the media were studied under varying concentrations of oxygen, air, carbon dioxide, and mixtures of these. In the presence of pure oxygen the rate of growth of *Brucella suis* was markedly increased over that obtained in a constant flow of air. In media containing low concentrations of tryptose the growth of *B. suis* was not significantly increased, but utilization of glucose was considerably greater. The growth of *Brucella abortus* was inhibited in the presence of small amounts of pure oxygen. In the presence of proper constituents of the medium and oxygen, *B. suis* utilized 1 per cent glucose for growth energy in 48 hours. Glucose was utilized to a very slight extent, if at all, in the absence of thiamine. There is no tendency for dissociation of *Brucella* under the conditions of this study, nor is there any change in virulence for experimental animals.

G37. *Microrespiration Studies with Fungi.* WILLIAM W. DORRELL AND S. G. KNIGHT, University of Wisconsin, Department of Agricultural Bacteriology, Madison 6, Wis.

The respiratory activity of *Fusarium graminearum* and *Penicillium notatum* was investigated by microrespiration techniques. Methods were developed for adapting fungus mycelium to such studies; the disadvantages of the characteristically high endogenous respiration rate were reduced to a great extent. By mincing mycelial tissue from a shake culture in a modified "blendor," a homogeneous suspension that was easily handled and quite active could be prepared. With the *Fusarium*, the total respiratory activity of the minced tissue was greater than that of whole mycelium although the response to added substrate was not enhanced. With the *Penicillium*, mincing reduced endogenous activity, but the response to added substrate was much greater than with the whole mycelium.

The starvation technique of storing active cells in a buffer at a low temperature for several days was investigated. With the *Penicillium*, the originally high endogenous rate was greatly reduced by such a procedure without reduction of exogenous activity. With the *Fusarium*, the endogenous rate was likewise reduced, but the response to added substrate was not great. The high endogenous rate could be reduced faster by aerating the cell suspension in a buffer or nutrient salt solution for a short time. In 24 hours, the endogenous respiration could be

halved while the respiratory response to substrate remained high. Both organisms responded alike to this procedure. By growing the *Fusarium* in a synthetic medium containing a low glucose content and a C/N ratio of 1.0 or lower, cells could be produced which showed an excellent response to substrate without the necessity of storage or aeration.

G38. *Studies on the Surface Lipids of Micrococcus aureus.* M. T. DYAR, Cornell University, College of Agriculture, Laboratory of Bacteriology, Ithaca, N. Y.

Dyar and Ordal observed that the electrophoretic mobility of *Micrococcus aureus*, grown in glucose broth, was greater when the cells were suspended in buffer solutions containing anionic surface-active agents than when in buffer alone. Comparison with known surfaces led them to suggest that the material in the cell surface responsible for this increase might be a lipid. This has been confirmed in the present study, and some of its properties have been investigated. Its presence is detected by an increase in the negative mobility of cells suspended in suitably buffered solutions of anionic surface-active agents, and its relative amount may be estimated by the magnitude of the change in mobility. This substance is completely removed from the surface of the cells, both live and heat-killed, by a purified preparation of pancreatic lipase. The occurrence of the lipid in the cell surface is favored, at all stages of cultural development, by growth in glucose broth with aeration, but traces also occur in cells grown in nutrient broth without aeration. Cells containing the lipid material exhibit some similarities in electrophoretic behavior to the fats extracted from cells of the same culture. An increase in this surface material is accompanied by an increase in polar groups, indicating the presence of phospholipids. The surface lipid is almost completely removed by extraction with hot alcohol; however, it appears to be firmly bound in the structure of the cell surface because it is not removed by prolonged ether extraction.

G39. *Studies on the Death of Bacteria by Drying. I. The Influence of in Vacuo Drying from Frozen State and from the Liquid State on the Initial Mortality and Storage Behavior of Escherichia coli.* RUSSELL S. WEISER AND LARS A. HENNUM, University of Washington, Department of Microbiology, Seattle, Wash.

The organisms from 20-hr nutrient agar cultures of *Escherichia coli* were washed and suspended in different menstrua for drying. The menstrua were 1 per cent peptone, pH 7.0, inactivated rabbit serum, and skim milk. The suspensions were subjected to *in vacuo* drying from the frozen state at  $-15^{\circ}\text{C}$  and from the liquid state at  $22^{\circ}\text{C}$ . They were sealed under vacuum and stored at room and refrigeration temperatures for intervals of a few weeks to several months. Plate counts made immediately after drying showed the initial mortality to be about 10 to 15 per cent greater in samples dried from the liquid state than in those dried from the frozen state. The initial mortality in the suspensions in peptone ranged from about 85 to 95 per cent, whereas the mortality of organisms suspended in

inactivated rabbit serum ranged from about 50 to 65 per cent. The mortality of organisms suspended in skim milk was intermediate but highly variable. Storage death studies showed the "half life" of survivors to be about 10 days for samples stored at room temperature and 40 days for those stored at 10 C. Preliminary trials indicate that the death rate was considerably slower at subfreezing storage temperatures than at higher temperatures.

G40. *Nutritional Requirements of Microbacterium lacticum*. RAYMOND N. DOETSCH AND MICHAEL J. PELCZAR, JR., University of Maryland, Department of Bacteriology, College Park, Md.

Studies on the vitamin requirements of 14 strains of *Microbacterium lacticum* Orla-Jensen were conducted. It was found possible to grow these organisms in a medium of the following composition: glucose (1.0 per cent); vitamin-free, salt-free casein hydrolyzate (0.5 per cent); *L*-asparagine (0.025 per cent); *dl*-tryptophane (0.01 per cent); *L*-cystine (0.001 per cent); *d*-calcium pantothenate (1.0 mg per ml); and inorganic salts. Pantothenate was an absolute requirement for growth; omission of this vitamin from the ingredients above resulted in a medium which failed to support growth of any of the strains. Other vitamins and growth factors were investigated to determine their influence upon the growth of *M. lacticum*. Thiamine and biotin appeared to be stimulatory when incorporated into the medium described above. Comparisons were made of the total growth obtained in this medium and in yeast extract peptone glucose broth.

G41. *The Calcium Requirements of Purple Bacteria, Green Algae, and Spirodela polyrrhiza*. S. H. HUTNER, Haskins Laboratories, New York 17, N. Y.

Calcium is considered essential for transfer of high-energy phosphate, yet its indispensability for microbial growth is uncertain. The Ca requirement of several microorganisms was investigated, using media assembled largely from components purified by distillation or solvent partition. With appropriate additions of Ca, such media supported heavy growth of purple bacteria, green algae, and the higher green plant *Spirodela polyrrhiza*, grown aseptically. One such medium had the following percentage composition: 0.05 KCl, 0.08 Na<sub>3</sub> citrate·2H<sub>2</sub>O, 0.08 (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.25 NH<sub>4</sub> succinate, 0.06 NH<sub>4</sub> acetate, 0.5 mg Fe (Westinghouse "puron"), 0.05 mg boron, plus the thiamine, nicotinic acid, *p*-aminobenzoic acid, and biotin required by purple bacteria; pH 6.8 to 7.1.

Exploratory experiments with media not as low in Ca as eventually contemplated revealed, nevertheless, great variation in Ca requirements. None was evident for *Rhodospseudomonas palustris*, *Rhodospseudomonas spheroides*, or *Chlorella*. *Scenedesmus* did not grow without added Ca, and attained full growth with 0.01 mg per cent. For maximal growth, *Rhodospirillum rubrum* required 0.1 mg per cent, *Rhodospseudomonas capsulatus* 0.8 mg per cent, and *Spirodela* 6.0 mg per cent Ca. Calcium was not replaceable by assortments of rare earths and many other elements. Calcium compounds purified to remove rare earth elements and other likely contaminants have not yet been assayed. Estimation of the avidity of organisms in acquiring Ca and of their absolute Ca requirements



(and, by extension, other trace elements) appears possible through determining the Ca requirement as a function of concentration of citrate or other complex former, and extrapolating to zero citrate.

*G42. Relation of Biotin to the Formation of Aspartic Acid by Microorganisms.*

J. L. STOKES, ALMA LARSEN, AND MARION GUNNESS, Merck & Co., Inc.,  
Department of Research and Development, Rahway, N. J.

*Streptococcus faecalis* R, *Lactobacillus casei*, *Lactobacillus arabinosus*, and related bacteria will not develop in culture media unless supplied with approximately 1 millimicrogram of biotin and 1 mg of aspartic acid per 10 ml of medium in addition to other required nutrients. An increase in the biotin content of the medium to 10 millimicrograms of biotin per 10 ml results in full growth in the absence of aspartic acid, and the growth can be serially subcultured in the same medium. Aspartic acid is synthesized in normal amounts by cells grown with excess biotin: for example, cells of *S. faecalis* grown with 50 millimicrograms of biotin per 10 ml of aspartic acid-free medium contained 5.5 per cent aspartic acid, whereas cells grown with aspartic acid contained 5.4 per cent of this amino acid. *o*-Heterobiotin and the diamino acid derivative of biotin are also effective in substituting for aspartic acid although much larger quantities of these as compared to biotin are required. Resting cell suspensions of *L. arabinosus* in M/15 phosphate buffer at pH 7 can form aspartic acid from mixtures of glutamic acid plus oxalacetate, and glutamic acid plus fumarate, or malate or succinate. Aspartic acid is also formed from alanine plus oxalacetate. However, biotin does not appear to take part in any of these reactions.

*G43. The Function of Pantothenic Acid in Bacterial Metabolism.* G. DAVID

NOVELLI AND FRITZ LIPMANN, Massachusetts General Hospital, Biochemical Research Laboratory, Boston, Mass.

Studies on acetylation in animal tissues led to the isolation of a new coenzyme (coenzyme A) which recently was characterized as a pantothenic acid (p.a.) derivative. Coenzyme A was found to be a general constituent of living organisms. It is present in plant and in animal tissues and in all microorganisms tested including species of *Lactobacillus*, *Propionibacterium*, *Escherichia*, *Proteus*, and *Clostridium*, the latter being particularly rich in coenzyme A. Earlier observations with *Proteus morganii* had linked p.a. to pyruvic acid metabolism. A stimulation of pyruvic acid oxidation by pantothenic acid in p.a.-deficient organisms was confirmed. The following observation suggested that such stimulation is due to synthesis of coenzyme A. In our experiments up to 90 per cent increase in pyruvate oxidation went parallel with up to 4-fold in coenzyme A content. In *Lactobacillus arabinosus* 90 per cent of the pantothenic acid could be accounted for as coenzyme A. Pantothenic-acid-deficient organisms contain negligible amounts of coenzyme A, and on suboptimal levels of p.a., coenzyme A concentration becomes a function of the p.a. level. It appears probable that, at least, much of the p.a. in living organisms is present as coenzyme A.

G44. *The Effect of Large Amounts of Nicotinic Acid and Nicotinamide on Bacterial Growth.* STEWART A. KOSER AND GEORGE J. KASAI, University of Chicago, Department of Bacteriology and Parasitology, Chicago 37, Ill.

To determine the effect of large amounts of a vitamin, the concentration of nicotinic acid and nicotinamide was increased many times over that needed to support optimum growth. The amount of vitamin was raised to 1,000  $\mu\text{g}$  per ml before any distinctly retarding effect on growth became apparent. In the presence of 3,000, 5,000 and 10,000  $\mu\text{g}$  per ml, growth was progressively retarded and often completely inhibited at the 10,000- $\mu\text{g}$  level. A specially purified sample of nicotinic acid gave results comparable to commercial samples. The organisms used included some which need preformed nicotinic acid and others which are able to synthesize the vitamin: several types of *Shigella paradysenteriae*, *Escherichia coli*, *Salmonella aertrycke*, *Salmonella enteritidis*, *Proteus vulgaris*, *Proteus morganii*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Staphylococcus albus*, and *Bacillus subtilis*.

The inhibitory effect of large amounts of the vitamin is more marked in a simple synthetic medium than in a casein digest medium. The inhibitory effect of 10,000  $\mu\text{g}$  is nullified by yeast extract, but not by a mixture of known vitamins. Organisms inhibited by 10,000  $\mu\text{g}$  may be carried through many successive transfers in the presence of 5,000  $\mu\text{g}$  nicotinic acid but the cells appear abnormal on microscopic examination.

G45. *Accessory Factor Requirement for Pyruvate Oxidation.* D. J. O'KANE AND I. C. GUNSALUS, College of Agriculture, Cornell University, Laboratory of Bacteriology, Ithaca, N. Y.

With the growth and test conditions suggested by Miller *et al.*, the nutritional factors influencing pyruvate oxidation by a strain of *Streptococcus faecalis* have been studied. A defined medium composed of accessory factors, buffer, glucose, enzyme-hydrolyzed casein, and acid-hydrolyzed casein supports growth equal to that obtained in the usual yeast extract tryptone medium, but the resting cells harvested from this medium oxidize pyruvate very slowly ( $Q_{O_2}$  (N) = 25). If the synthetic medium is supplemented with yeast extract, or if yeast extract is added to resting cells from the synthetic medium, pyruvate is oxidized rapidly ( $Q_{O_2}$  (N) = 400). This requirement for pyruvate oxidation is somewhat specific, since the deficient cells oxidize glucose rapidly ( $Q_{O_2}$  (N) = 600). These results indicate that the nutritional requirements for pyruvate oxidation are more specific than those for growth, and that the enzymes necessary for pyruvate oxidation are preformed in the cells. Various known factors, such as folic acid, purines, etc., singly and in combinations, have not been found to replace yeast extract. Several other natural materials such as brewers' and bakers' yeast and various liver fractions are, however, active.

Although sufficient data are not available to characterize the active material, some properties have been determined. Autoclaving for 1 hour in 4 N sulfuric acid or for 30 minutes in 1 N sodium hydroxide does not destroy the activity. It is not extracted from aqueous solution by ether, but is partially removed

by butyl alcohol at pH 7. Mercury precipitates the active fraction, but basic lead acetate and picric acid do not. Partial purification has been achieved by adsorption or charcoal followed by elution.

G46. *Pyridoxal Phosphate as the Coenzyme of Tryptophanase from Escherichia coli.* W. AVERY WOOD, I. C. GUNSALUS, AND W. W. UMBREIT, College of Agriculture, Cornell University, Laboratory of Bacteriology, Ithaca, N. Y.

*Escherichia coli* cells which contain a very active enzyme system for the formation of indole from tryptophane have been obtained by growing the culture with aeration in a medium containing tryptone, yeast extract, phosphate, and 0.1 per cent glucose. These suspensions have been vacuum- or acetone-dried to yield cell preparations which contain most of the tryptophanase activity present in the living cells. This enzyme is stable in the dried preparations and the amount can be estimated by determining the rate of indole formation from tryptophane in M/10 phosphate buffer at pH 8.3. Indole is determined colorimetrically by a quantitative modification of Ehrlich's method. Tryptophanase has been extracted from autolyzed acetone-dried cells in a cell-free state. The resolution and partial purification have been accomplished by precipitation of the extracts with ammonium sulfate and by adsorption on calcium phosphate gel. After dialysis, the water-clear enzyme solution is completely resolved. Pyridoxal phosphate will reactivate these enzyme solutions, thereby adding tryptophanase to the group of vitamin B<sub>6</sub> enzymes. The products of tryptophanase action are indole, pyruvic acid, and ammonia. Serine and alanine deaminases are present in the dried cells but absent from the cell-free enzyme; therefore these do not appear to be intermediates in the formation of pyruvate. In the cell-free system no oxygen is taken up. The reaction is retarded by the accumulation of indole and is inhibited by cyanide. Although tryptophanase bears a superficial resemblance to the *Neurospora* enzyme which synthesizes tryptophane from indole and serine, the two are not identical.

G47. *The Relation of Certain Amino Acids to the Rate of Glycolysis of Lactic Acid Bacteria.* C. E. FOUST AND I. C. GUNSALUS, College of Agriculture, Cornell University, Laboratory of Bacteriology, Ithaca, N. Y.

The rate of glycolysis by resting cell suspensions and by dried cell preparations of lactic acid bacteria can be accelerated by the addition of yeast extract. Prolonged autoclaving with strong acid does not reduce the effectiveness of the yeast extract. Fractionation of the acid autoclaved yeast extract with butyl alcohol and silver has resulted in the separation of two active fractions which can be replaced by histidine and glutamic acid. Addition to the glycolysis system of histidine and glutamic acid combined in a molar ratio of one to three, together with ammonia, will reproduce the acceleration in rate observed with yeast extract. The action of these amino acids on glycolysis does not appear to be the stimulation in rate of any single step in the glycolytic system. When glucose and fructose are used as substrates, the rate accelerates upon addition

of yeast extract or the amino-acid-ammonia combination, but when the substrate is one of the phosphorylated ester intermediates, no stimulation is observed. Similarly, dried cell preparations in which glycolysis is inhibited by iodoacetate will effect a transformation of phosphorus from adenosine triphosphate to glucose or fructose at a measurable rate, but the rate is not accelerated by addition of the factor.

G48. *Pyruvic Acid Metabolism of Streptococci*. A. KATHRINE MILLER, J. J. R. CAMPBELL, AND I. C. GUNSALUS, College of Agriculture, Cornell University, Laboratory of Bacteriology, Ithaca, N. Y.

The importance of pyruvic acid as a metabolic intermediate, and the several routes of pyruvate breakdown even in homofermentative lactic acid bacteria, have led to a study of the influence of cultural conditions upon the enzyme complement of *Streptococcus faecalis*, strain 10Cl, for this substrate. Rapid pyruvate oxidation is obtained with cells harvested from a medium containing yeast extract, tryptone, 0.5 per cent phosphate, and 0.3 per cent glucose; final pH about 6. Cells from 12- to 15-hour cultures at 37 C oxidize pyruvate rapidly, whereas older cultures, or those harvested from more acid or more neutral media, do not oxidize pyruvate so rapidly. Since the optimum pH for pyruvate oxidation is around 6.5, this system belongs to the class of enzymes formed in maximal amount near the optimum pH for activity. The cultural conditions which produce cells with strong pyruvate-oxidizing ability also favor the dismutation of pyruvate to lactate, acetate, and CO<sub>2</sub>. Cells grown in acid medium, or with oxidized substrates, form small amounts of acetylmethylcarbinol, but this reaction does not compete well with pyruvate dismutation in resting cells unless inhibitors are present. In the presence of arsenite, and acid reaction, appreciable amounts of acetylmethylcarbinol are formed from pyruvate. Cells harvested from neutral media or media containing citric acid form appreciable amounts of formic and acetic acids at a pH above 7. These resting cells oxidize or dismutate pyruvate slowly at alkaline reaction, yet are able to metabolize it at an appreciable rate, especially if extra thiamine is supplied in the medium.

G49. *Oxidation of Ethanol to Acetic Acid by Fluorescent Pseudomonads*. R. Y. STANTIER, Indiana University, Department of Botany and Bacteriology, Bloomington, Ind.

The ability to metabolize ethanol is a common, though not a universal, property of organisms belonging to the *Pseudomonas fluorescens* species group. Of those strains which can use ethanol, a majority produce substantial amounts of acetic acid from it in aerated cultures under favorable conditions. In a poorly buffered medium (e.g., peptone broth + 1.5 per cent ethanol) ethanol utilization is slight, owing to the rapid acidification which occurs; the pH drops to below 5.0 in 2 to 3 days, at which time development ceases. In a similar medium well buffered with CaCO<sub>3</sub>, practically complete utilization of the ethanol takes place in 4 to 5 days, with a concomitant accumulation of calcium acetate in amounts up to 50 per cent of theoretical amount. Taken in conjunction

with the recent discovery that fluorescent pseudomonads can oxidize monosaccharides to the corresponding -onic acids, this finding shows that a strictly biochemical separation between the morphologically similar genera *Pseudomonas* and *Acetobacter* is no longer possible. If the genus *Acetobacter* is still to be maintained, it must be not on biochemical but on physiological grounds, namely, a markedly greater acid tolerance than is found in other heterotrophic pseudomonads.

G50. *Replacement of Carbon Dioxide in Heterotrophic Metabolism.* SAMUEL J. AJL, A. G. C. WHITE, AND C. H. WERKMAN, Iowa State College, Bacteriology Section and Industrial Science Research Institute, Ames, Iowa.

Carbon dioxide is required by heterotrophic bacteria to initiate optimal growth and reproduction under normal environmental conditions. However, carbon dioxide may be replaced by intermediates of the Krebs cycle or compounds readily converted to such intermediates. Substances replacing CO<sub>2</sub> were: cis-aconitic,  $\alpha$ -ketoglutaric, succinic, fumaric, oxalacetic, glutamic, and aspartic acids, proline, and arginine. Pyruvic acid, acetic acid, lysine, and alanine were among those not replacing CO<sub>2</sub>. *Escherichia coli* and *Aerobacter aerogenes* were used. In the case of *A. aerogenes* citric acid did replace, whereas it did not with *E. coli*. The implications of the results are discussed.

G51. *The Influence of Oxygen on the Formation of Glycerol During Alcoholic Fermentation.* M. C. BROCKMANN AND T. J. B. STIER, Joseph E. Seagram & Sons, Inc., Louisville, Ky., and Indiana University, Bloomington, Ind.

It has long been recognized that the amount of glycerol formed during alcoholic fermentation can be altered by the fermentation conditions. In our experiments the oxygen tension either of the fermenting medium or of the yeast inoculum was shown to influence the production of glycerol in a glucose yeast extract KH<sub>2</sub>PO<sub>4</sub> medium maintained at 30 C. With inoculum developed in cotton-plugged flasks (initially aerobic) glycerol production per cell, as well as per unit of glucose metabolized, was slightly less in aerated medium than in medium held in cotton-plugged flasks or in medium sparged at the time of inoculation with inert gas containing less than 0.04 per cent oxygen. When yeast which had been developed under different oxygen tensions was used to inoculate media sparged with inert gas, the variations in glycerol production were more marked. Here glycerol production, following inoculation with yeast developed under aeration or in cotton-plugged flasks, was practically the same but considerably below that observed with yeast carried for several transfers under continuous sparging with CO<sub>2</sub>. Repetition of the preceding experiment in medium containing 0.002 M azide showed that on a per cell basis yeast developed under CO<sub>2</sub> sparging produced considerably more glycerol than yeast developed under aeration or in cotton-plugged flasks. These results are not obtained when glycerol production is calculated on the basis of glucose metabolized. With the three types of inocula, glycerol formation per cell runs parallel to the observed

activities of cell phosphatase. If phosphatase activity is a significant factor in the control of glycerol output per cell, it follows that the influence of oxygen on glycerol formation stems at least in part from its effect on cell phosphatase activity.

G52. *An Antibiotic from Bacillus subtilis Active Against Pathogenic Fungi.*

MAURICE LANDY, SANFORD B. ROSENMAN, AND GEORGE H. WARREN,  
Wyeth Institute of Applied Biochemistry, Department of Bacteriology,  
Philadelphia 30, Pa.

An antibiotic active against the important pathogenic fungi and possessing relatively negligible antibacterial properties has been obtained from *Bacillus subtilis*. The antibiotic is fungistatic in high dilutions and fungicidal (sporicidal) in greater concentrations. The active principle is readily produced in shallow layers (5 to 6 days) or shaker flask cultures (2 to 3 days) on a synthetic medium (glutamate-glucose inorganic salts) at 30 C. The antibiotic may be concentrated by precipitation from the culture broth at pH 2.5, the resultant precipitate extracted with ethanol and the activity precipitated by ether. The antibiotic concentrate thus isolated shows unusual heat stability, is not inactivated in a pH range of 2.5 to 9.0, and does not dialyze through cellophane membranes; and the active agent is not inactivated by body fluids. Complete inhibition of *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Microsporum audouinii*, *Microsporum gypseum*, *Trichophyton rubrum*, and *Trichophyton schoenleinii* was obtained in medium containing 0.025 mg per ml. *Blastomyces dermatitidis* (yeast form) is especially sensitive to the antibiotic, being completely inhibited by a concentration of 0.025 mg per ml, but the mycelial form required a concentration of 0.01 mg per ml to suppress its growth. On the other hand, *Nocardia asteroides*, *Philophora verrucosa*, and *Hormodendrum pedrosoi* are relatively resistant to its action. Complete growth suppression of *Candida albicans*, *Cryptococcus neoformans*, and *Sporotrichum schenckii* is obtained with 0.05 mg per ml. *Coccidioides immitis*, *Histoplasma capsulatum*, *Monosporium apiospermum*, and *Blastomyces brasiliensis* are slightly more sensitive, only 0.025 mg per ml being required for their complete inhibition.

G53. *Antibiotic Activity of Bacillus polymyxa.* R. G. BENEDICT AND A. F.

LANGLYKKE, Northern Regional Research Laboratory, Fermentation  
Division, Peoria, Ill.

In a survey of a large number of soil samples for antagonists active against members of the genera *Brucella* and *Mycobacterium*, several bacterial strains resembling *Bacillus polymyxa* were isolated. In various plate media, these strains inhibited the growth of a wide variety of gram-negative and gram-positive bacteria. Identity of these strains with known cultures of *Bacillus polymyxa* was established by certain physiological and spectrum plate tests. The active component is water-soluble and readily produced free from the bacterial cells. Highest yields in crude liquid culture have been obtained from corn steep glucose carbonate medium and asparagine mineral salts glucose me-

dium. Sterile culture filtrates inhibit *Brucella bronchiseptica* (NRRL B-140) in a dilution of 1:1,000. Experiments are in progress to produce the substance on a larger scale so that its physical and chemical properties may be compared with those of known antibiotics. We believe that this constitutes the first report in the literature of antagonistic activity by this organism.

*G54. Soil Actinomycetes of Bikini Atoll, with Special Reference to an Antibiotic-producing Organism.* DONALD B. JOHNSTONE, New Jersey Agricultural Experiment Station, Department of Microbiology, New Brunswick, N. J.

In connection with a study of the bacterial population of the waters around Bikini and Rongelap Atolls during the recent atomic bomb experiments in the Marshall Islands, a study of the microbiological population of the soil was conducted with samples obtained from various islands. A marked predominance of actinomycetes was noted. A number of these organisms were isolated and brought back to this country for further study. A special investigation was made of antibiotic properties of these cultures. One of the cultures, a species belonging to the genus *Streptomyces*, showed a marked inhibition of various bacteria including members of the genus *Mycobacterium*. The antibiotic substance produced by this organism resembles streptomycin in some respects, particularly with regard to its antimicrobial spectrum, but differs in certain other characteristics. Details of the methods of culturing the organism, extraction of the antibiotic substance, and its *in vitro* and *in vivo* activities will be presented.

*G55. A Capsule-dissolving Factor.* JAMES C. HUMPHRIES, University of Kentucky, Department of Bacteriology, Lexington, Ky.

Phage lysates of type A *Klebsiella pneumoniae* contain a factor which decapsulates cells both living and killed of type A Friedlander bacillus. This capsule lysine ("C" lysine) is independent of the phage: phage inactivation by formalin or heating (75 C) results in but slight reduction in the capsule-dissolving power of lysates. Filtration of lysates through collodion membranes of suitable porosity retain phage but allow passage of capsule-dissolving activity. The ultrafiltrates thus obtained are nontoxic to the growth of *Klebsiella*. That the capsule is actually removed is demonstrable by loss of serological type specificity, by reduction in cell volume, and by the acquired susceptibility of encapsulated cultures grown in the presence of the factor to lysis by a phage specific for nonencapsulated culture variants. "C" lysine is highly specific; it decapsulated all type A Friedlander cultures tested but was inactive against types B and C of *Klebsiella* and encapsulated *Aerobacter* strains. It is relatively heat-labile (85 C for 10 minutes), precipitable by ammonium sulfate, alcohol, and acetone, and nondialyzable. It has then many of the properties of enzymes. The substrate is unknown. Exposure of the capsular polysaccharide to "C" lysine does not result in any decrease in its serological activity, for when the encapsulated cells are treated with the lysine, antibody-precipitable SSS

is liberated into the medium, whereas similar treatment of chemically separated SSS causes no reduction in precipitin titer of the polysaccharide.

G56. *The Antagonism of Coliform Organisms Against Shigellae*. SEYMOUR P. HALBERT, University of North Carolina, School of Public Health, Chapel Hill, N. C.

Observations have been reported which have suggested that strains of *Escherichia coli* are capable of antagonizing a wide variety of microorganisms, including some of the intestinal pathogens, principally *Eberthella typhosa*. In the present study, 1,243 strains of coliform organisms (mostly *E. coli*) from 147 presumably healthy Latin-American children have been studied for their antagonistic activity on solid media against an arbitrarily selected strain of *Shigella* Flexner, type Z (III). Ten per cent of the coliforms produced zones of inhibition of the test organism from 2 to 8 mm wide; 7.8 per cent showed zones less than 2 mm; and 82.3 per cent were not antagonistic. Twelve to 20 sample strains of varying activity were tested against a wide variety of shigellae and salmonellae, as well as other gram-negative and gram-positive organisms, including *Neisseria catarrhalis*, *Vibrio comma*, *Klebsiella pneumoniae*, *Bacillus anthracis*, *Streptococcus faecalis*, *Staphylococcus albus*, and *Sarcina lutea*. All proved resistant, except 27 of the 46 strains of shigellae. The zones of inhibition are unaffected by as much as 25 per cent human serum or blood. The antagonism was demonstrated to be due to the production of a relatively heat-stable antibiotic which is readily absorbed on Seitz filters and charcoal. Crude supernatants from 6 active strains have inhibited in dilutions of 1:1,280 to 1:20,480 an inoculum of about 1,000 *Shigella* Z. Similar supernatants, from 2 strains, non-antagonistic on solid media, have been completely inactive, even in dilutions as low as 1:2.

G57. *A Turbidimetric Method for the Assay of Antibiotics*. DWIGHT A. JOSLYN AND MARGARET GALBRAITH, Parke, Davis & Company, Research Department, Detroit, Mich.

The purpose of a method for the assay of antibiotics is twofold: to determine the activity accurately and to do this quickly. One of the main reasons for a quick method is to be able to follow the potency of the antibiotic during its production in order that the harvest may be carried out at the peak of activity. These ends may be achieved by a turbidimetric method to a better advantage than by using the usual plate methods which require overnight incubation and a possible repeat, which adds 24 hours to the original determination. In this turbidimetric assay the end point is considered to be that concentration of the antibiotic which inhibits the growth of the test organism by 50 per cent. An important consideration in this test is the use of an actively growing culture in order that the growth curve may not show an appreciable lag phase. A rapidly growing culture is similar to the condition found in the clinic in the presence of an active infection. This method may be used to evaluate an unknown antibiotic or, with slight modifications, to evaluate one for which a standard has been established.



G58. *Anomalous Findings in Penicillin Level Determinations in Urines.*

CAROLYN R. FALK AND HELEN BLECH, Bureau of Laboratories, Department of Health, New York City, N. Y.

In the course of determining penicillin levels in urines of patients under treatment for syphilis, unexpectedly high levels were observed in most urines collected up to the fourth hour after drug administration. Since these results were higher than could be explained by the dose given, some of the factors influencing the test were studied. All specimens were refrigerated from time of collection, altho a delay of several days until time of assay was frequent. In the initial tests, 7 of 12 specimens had levels from 5 to 1,000 times higher than was anticipated. During a period of 5 weeks' storage, another 7 of 12 specimens showed an increase of from 10- to 100-fold times that of the original reading. Only one specimen failed to show significantly increased bactericidal action at some time during this period. Similar results could not be reproduced by adding up to 10,000 O.U. per ml to normal urine. Likewise, an increased penicillin content was not found in the urines, of the same patients, collected over 4 hours after drug injection. Moreover, the levels of specimens stored more than 5 weeks decreased and were comparable to the dose administered. No explanation for this phenomenon has been found. It is presented to call attention to irregularities that may be encountered in attempting to make a quantitative estimate of the penicillin content of early urines, particularly when tests are delayed.

G59. *Antibiotic Interrelationships Among the Enteric Group of Bacteria.* PIERRE

FREDERICQ AND MAX LEVINE, Iowa State College, Department of Bacteriology, Ames, Ia.

The observations of Gratia (1925-32) and his associates on antibiotic agents produced by coliform bacteria were confirmed and extended. Coliform strains were encountered which exhibited antibiotic effects against some of the Flexner as well as the Shiga and Sonne dysentery strains. The autoinhibitory phenomenon reported by Powers and Levine and Coblentz and Levine among members of the coliform group of bacteria appears to be different from the antibiotic effects under consideration. The antibiotic spectra of various active strains appear to be specifically characteristic. That there may be some correlation of biochemical and antigenic structure with antibiotic sensitivity is indicated by the observation that when a strain of a well-defined type of *Salmonella* or *Shigella* was found to be sensitive to one or more cultures, all other strains of the same type available at the time of this study were likewise susceptible to the antibiotics produced by those active cultures.

G60. *Isolation of Streptomycin-producing Strains of Streptomyces griseus.* H.

CHRISTINE REILLY, New Jersey Agricultural Experiment Station, Department of Microbiology, New Brunswick, N. J.

The problem of isolation of streptomycin-producing cultures of *Streptomyces griseus* comprises two approaches: (a) the isolation of fresh cultures from the natural environment; (b) the isolation of more active strains from known cul-

tures. The latter becomes of particular importance in the commercial production of streptomycin, since the culture tends to lose its ability to produce this antibiotic. When an active culture is plated out on a suitable agar medium and a number of colonies picked, the new strains thus obtained were found to vary considerably in the production of streptomycin. Some produce very little or no active material, whereas others produce as much as or even more than the parent strain. The presence of 50  $\mu$ g of streptomycin per ml of agar, used for isolation purposes, does not necessarily favor the isolation of strains with high activity. Among the substrains obtained, two streptomycin-producing cultures were found to produce a high activity in submerged culture but comparatively little activity in stationary culture, a phenomenon not observed in the original streptomycin-producing culture from which these strains were isolated.

G61. *A Quick Microtechnique for the Detection of the Reduction of Nitrates to Nitrites by Bacteria.* BARBARA BACHMANN AND R. H. WEAVER, University of Kentucky, Department of Bacteriology, Lexington, Ky.

The principles of Weaver, Arnold, and Hannan (1946) have been used in the development of a quick microtechnique for the detection of the reduction of nitrates to nitrites by bacteria. The performance of the entire test, including the growth of cultures for inocula, requires slightly more than 6 hours. The medium, nitrate broth containing 1 per cent peptone, is dispensed in 10  $\times$  75 mm tubes in 1-ml quantities and preheated in a 37 C water bath. The tubes are removed from the bath and inoculated with two loopfuls of growth from 6-hour 1 per cent peptone nutrient agar slant cultures. The tubes are returned to the water bath for 15 minutes, after which time they are removed and tests are performed for the presence of nitrite by the addition of one drop each of solutions of sulfanilic acid and dimethylalphanaphthylamine, both in acetic acid. Results which correlate perfectly with those of the routine test for nitrate reduction have been obtained with 757 freshly isolated cultures. It is hoped that by modifying the medium used to suit the growth requirements of various organisms this test can be applied as extensively as is the routine test. It is possible that the length of the incubation period may have to be increased for cultures that have slow growth rates.

G62. *A Quick Microtechnique for the Detection of Hydrogen Sulfide Production by Bacteria.* M. L. MORSE AND R. H. WEAVER, University of Kentucky, Department of Bacteriology, Lexington, Ky.

The principles of Weaver, Arnold, and Hannan (1946) have been used in the development of a quick microtechnique for the detection of hydrogen sulfide production by bacteria. A 2 per cent thiopeptone solution, pH 6.8, is dispensed in 0.8-ml amounts in 10  $\times$  75 mm tubes. The tubes are preheated to 37 C, and each is then inoculated from a 6-hour infusion agar slant culture to give a turbidity corresponding to approximately 2,100 million organisms per ml. A strip of lead acetate paper is inserted in the mouth of the tube, and the tube is

returned to the bath. A comparison has been made between the results obtained by the microtechnique and those obtained by the use of Dunham's peptone solution with lead acetate paper, iron peptone agar, and lead acetate agar on 798 cultures (mostly *Enterobacteriaceae*). Of 275 cultures that gave positive results with all three comparison media, 86 per cent gave positive results with the microtechnique in 30 minutes and 98.5 per cent in 45 minutes. Of 211 cultures that gave positive results with one or two of the comparison media, 32 per cent gave positive results with the microtechnique in 30 minutes and 55 per cent in 45 minutes. Of 312 cultures that gave negative results with the three comparison media, 3.5 per cent gave positive results with the microtechnique in 30 minutes and 10.5 per cent in 45 minutes. As shown by previous workers, the results of hydrogen sulfide tests vary with the medium and the method used for demonstration.

G63. *Acid Production from Glucose in the Genus Actinomyces*. VINCENT W. COCHRANE, Connecticut Agricultural Experiment Station, Department of Botany and Plant Pathology, New Haven, Conn.

The production of acid by *Actinomyces* is of value in characterization. Over 100 named species and unidentified soil strains were grown on glucose asparagine agar plates with bromocresol purple. About 50 per cent were strongly positive, forming acid within 4 days; about 20 per cent were negative. The remaining cultures produced acid late in the growth cycle and could not be characterized by this criterion. Members of the strongly positive and the negative groups were easily differentiated. Representative species were grown in a glucose asparagine yeast-extract medium in aerated culture; growth, sugar utilization, and acid formation were measured over the period of growth. These data show that the grouping of a particular isolate by its reaction on indicator agar is correlated with the accumulation in liquid medium of ether-extractable organic acids. *Actinomyces coelicolor*, which was strongly positive on indicator agar, produced an acidity equivalent to 0.02 N; at a comparable stage in the growth cycle, *Actinomyces reticuli*, negative on indicator agar, produced no significant amount of acid. Volatile acids were not present in aerated cultures.

G64. *Studies in the Biochemistry of Neurospora*. AARON E. WASSERMAN AND BERNARD S. GOULD, Massachusetts Institute of Technology, Department of Biology, Cambridge, Mass.

The method is essentially that of Doermann for assay of lysine but has been modified in that glucose has been substituted for sucrose in the assay medium and, instead of measuring growth dry weights after 7 days' incubation, the glucose consumption after 3 days' growth is determined by copper reduction methods. Using the shorter method, the utilization of glucose appears proportional to the lysine content of the medium. Using the wild strain of *Neurospora crassa* as well as the lysineless strain no. 4545, it has been found that while glucose is fermented at only a slightly more rapid rate than fructose with mixtures of the two sugars or with invert sugar arising from the hydrolysis of sucrose,

glucose is preferentially fermented. *Neurospora* is in this respect similar to most yeasts, except a sauterne yeast which ferments fructose preferentially. This suggests a probable similarity in the mechanisms of carbohydrate metabolism. Glucose inhibits and fructose enhances formation of invertase by *Neurospora*. Sugars which on hydrolysis yield glucose also inhibit enzyme production, whereas those yielding fructose, but not glucose, enhance enzyme production; thus sucrose inhibits whereas raffinose enhances invertase production.

G65. *An Unusual Strain of Pseudomonas aeruginosa*. LEWIS H. SCHWARZ AND JOSEPH A. LAZARUS, J. A. Lazarus, 875 Park Avenue, New York 21, N. Y.

The only reference to a mucoid encapsulated form of *Pseudomonas aeruginosa* is by Sonnenschein (1896). An organism exhibiting striking mucoid colonies on blood agar plates was recovered from the bladder urine of a patient who had had a kidney removed for a calculus pyelonephrosis. Cultural studies showed this organism to be *Pseudomonas aeruginosa*. Upon primary isolation a capsule could be clearly demonstrated. The organism was gram-negative, fermented glucose slightly, although the other sugars were not attacked, and was extremely proteolytic. Gelatin was rapidly liquefied, as was Loeffler's serum. The pigment produced was pyocyanin, whereas pyorubrin was found in older cultures. The organism was extremely pathogenic, showed strong toxic manifestations, and elaborated an endotoxin and an exotoxin. This organism was subsequently isolated on several occasions from the same patient, and similar strains from other patients with urinary tract infections. This variant is more virulent than other strains of *Pseudomonas aeruginosa* studied. Pigment production is not pronounced. The organism is more resistant to the action of streptomycin than are other members of this group. Serum obtained from carriers agglutinated the organisms in a titer of 1:5,120, as compared to 1:10 and 1:20 in a series of ten normal controls.

G66. *Cultural Studies of the Yeastlike Phase of Histoplasma capsulatum Darling*. S. B. SALVIN, National Institute of Health, Division of Infectious Diseases, Bethesda, Md.

The yeastlike phase of 17 different strains of *Histoplasma capsulatum* Darling was grown in a fluid medium. The cultural requirements of the organism were examined in the attempt to obtain maximum growth.

G67. *The Influence of Mechanical Handling upon the Growth of Single Bacterial Cell Isolates*. W. E. GRUNDY AND J. J. REID, The Pennsylvania State College, Department of Bacteriology, State College, Pa.

The effect of mechanical handling of single bacterial cells upon their ability to grow has been investigated. The Chamber micromanipulator was employed using the technique of Hildebrand. The percentage of growth of single cells which were picked up and dropped several times was compared with control cells handled only once. The following organisms, a *Bacillus danicus*, *Bacillus*

*anthracis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae*, respectively, were investigated. At least 50 single cells of each species were isolated. The additional mechanical handling beyond the normal manipulation had no effect with any of the organisms. A high percentage of growth was obtained in all cases. The mechanical feature of micromanipulation has apparently been overemphasized.

G68. *The V-Factor Content of Stored Blood.* WENDELL GINGRICH, University of Texas Medical School, Department of Bacteriology, Galveston, Texas.

Because stored blood has occasionally proved unsatisfactory for the preparation of media for *Hemophilus influenzae* and because the posttransfusion survival of red cells is poor under certain conditions, the V-factor was studied. Blood stored for various periods of time was extracted by water at 90 C for 10 minutes and compared with standards of codehydrogenase I for support of growth of *Hemophilus parainfluenzae*. There did not appear to be sufficient decrease of V-factor in any blood tested to account for either of the difficulties mentioned.

G69. *X-Ray-induced Mutations of Actinomyces flaveolus.* ALBERT KELNER, Biological Laboratory, Cold Spring Harbor, N. Y.

During an investigation of X-ray-induced mutations of microorganisms, a representative saprophytic actinomycete species, *Actinomyces flaveolus*, was subjected to X-rays. Suspensions of the conidia in saline were treated with doses ranging from 25,000 to 300,000 roentgen units. The conidia proved relatively resistant to the killing effect of X-rays; in one experiment 6 per cent survived 100,000 r units, and 0.03 per cent 300,000 r units. The survival rate was inversely proportional to the dose. Among the survivors of irradiated suspensions were found the following types of mutants: biochemically deficient strains which grew well on nutrient agar, but very poorly or not at all on asparagine glucose agar; strains with more intense or with different pigmentation than the wild type; and asporogenous strains. The percentage of such mutants among the survivors increased as the X-ray dose was increased. About 24 per cent of the survivors of a suspension treated with 200,000 r units were mutants.

A1. *Missed Contaminations in Biologic Products: The Role of Cryophile Bacteria.*

Geoffrey Edsall and Leslie H. Wetterlow, Division of Biologic Laboratories, Massachusetts Department of Public Health, Jamaica Plain (Boston), Mass.

The purpose of this report is to describe and stress the significance of bacterial contaminants which are not detected by incubation at 35 to 37 C. Contaminants of this type have been shown, by us and others, to produce pyrogenic substances profusely, and are frequently found in pyrogenic lots of serums. Their detection and their elimination are therefore of major importance. During the past 7 years, all sterility tests at this laboratory have been performed at room temperature (20 to 25 C) as well as at 35 to 37 C. A number of products

have been found contaminated with gram-negative bacilli which would grow only at the lower temperature range. During the sample years, 1942 and 1946, for example, out of 65 proven contaminations, 21, or 32 per cent, were due to such "thermophobic" bacilli. The organisms of this type isolated so far have been capable of growth from near 0 C to about 33 C, but grow best at 20 to 25 C. They vary in many detailed characteristics, but in general resemble the genus *Pseudomonas*. Similar strains have been isolated by us from air and water. Detection is frequently difficult, particularly if product contains no preservative. Detection has been aided by leaving samples for culture at room temperature overnight before planting, by taking larger than usual samples, and by prolonging the period of observation of the tests.

*A2. Problems Involved in the Sterility Testing of Nonaqueous Pharmaceutical Preparations.* JOHN H. BREWER, Hynson, Westcott & Dunning, Inc., Biological Research, Baltimore, Md.

Many pharmaceutical preparations are not soluble or readily miscible in culture media and cannot be properly tested by the usual sterility test methods. This is particularly true of powders and oils. Methods for insuring the sterility of these preparations are discussed and techniques of testing are described. Special attention is given to methods of preparing spore suspensions for use as controls. Spores are placed in polyethylene glycol, which will withstand high temperatures and, unlike oil, is miscible with culture media.

*A3. The Inactivation of Penicillin with Hydroxylamine and Its Use in the Sterility Testing of Penicillin.* W. A. RANDALL, B. A. LINDEN, AND H. WELCH, Food and Drug Administration, Division of Penicillin Control and Immunology, Washington, D. C.

Hydroxylamine at a pH near 7.0 rapidly and completely inactivates penicillin. Since all of the known penicillin species are inactivated, the point of action must be on that part of the molecule common to all species. The reaction products resulting from the complete inactivation of penicillin with hydroxylamine were not inhibitory for numerous species of bacteria from representative genera when fluid thioglycolate broth was used as the recovery medium. When small quantities of the various organisms in a liquid menstruum were added to dry penicillin powder (amorphous) and the penicillin inactivated with hydroxylamine, only about two-thirds of the species tested could be recovered in fluid thioglycolate medium. When, however, these same organisms were added in a dry state, all could be recovered following inactivation of the penicillin. On the basis of these findings a sterility test technique for penicillin was developed which has been used by the Food and Drug Administration for several years with satisfactory results.

*A4. The Production, Isolation, and Utilization of Penicillinase.* J. F. MORGAN AND M. E. CAMPBELL, Laboratory of Hygiene, National Health and Welfare Department, Ottawa, Ont.

Production of the adaptive extracellular penicillinase by the gram-positive sporeforming organism NRRL 569 was investigated with particular reference to the cultural conditions influencing enzyme formation. Variation in lots of yeast extract, pH during growth of the organism, presence of fermentable carbohydrate, and response to penicillin addition were found to affect penicillinase yield. By proper adjustment of these critical factors high enzyme titers were reached in 24 to 36 hours, as compared with the 5-day period previously found necessary. Methods of isolation were investigated in an effort to simplify the previously reported purification procedure based on adsorption and elution techniques. Recovery of penicillinase from the filtrate of the culture was achieved by isoelectric precipitation in the range of pH 5.4 to 5.0. Ammonium sulfate fractionation of this precipitate gave high yields of active material. By this method relatively pure enzyme was obtained with ease and rapidity. The sterility testing of penicillin has been investigated in regard to inactivating agents and to more suitable culture media. Purified penicillinase was shown to be superior to clarase, hydroxylamine, or cysteine. Two types of medium were found advisable in routine sterility tests. Several lots of antibiotics were detected as contaminated when tested in a modified beef heart medium although no growth was evident in thioglycolate medium.

*A5. The Antifungal and Antibacterial Action of Dibromsalicylaldehyde.* C. BAXTER McLAUGHLIN AND JOHN H. BREWER, Hynson, Westcott & Dunning, Inc., Biological Research, Baltimore, Md.

Dibromsalicylaldehyde has been found to be active against the common pathogenic fungi and sufficiently nontoxic to be used against infections due to these organisms. This compound is particularly effective against *Pseudomonas aeruginosa* and other gram-negative bacteria. The antifungal properties of dibromsalicylaldehyde are compared to those of several fatty acids and their salts. A method for the preparation of uniform suspensions of fungi, for use in testing antifungal activity, is described.

*A6. Iodonium Compounds and Their Antibacterial Activity.* LOUIS GERSHENFELD AND BERNARD WITLIN, Philadelphia College of Pharmacy and Science, Department of Bacteriology, Philadelphia, Pa.

The iodonium compounds are members of a class of compounds in which iodine is present as an integral part of their structure as positive ions. They have only recently been studied from the standpoint of their practical value. Several iodonium compounds were tested. Antibacterial efficiency tests were conducted with diphenyl iodonium chloride, bis-*p*-chlorophenyl iodonium sulfate, bis-*p*-bromophenyl iodonium iodide, bis-*p*-chlorophenyl iodonium iodide, bis-*p*-iodophenyl iodonium iodide, and diphenyl iodonium iodide. (1) These iodonium compounds (in powder form) displayed bacteriostatic activity when tested by the FDA agar plate technique. (2) Only bis-*p*-chlorophenyl iodonium sulfate in a saturated aqueous solution (even diluted 1:4) displayed bactericidal efficiency against *Staphylococcus aureus* at 37 C within 1 minute. (3) Satu-

rated solutions of all the iodonium compounds in a solvent consisting of 10 per cent acetone by volume in alcohol (95 per cent) displayed bactericidal efficiency against *Staphylococcus aureus* at 37 C within 1 minute. (4) A saturated alcohol-acetone solution of bis-*p*-chlorophenyl iodonium sulfate was capable of killing *Bacillus subtilis* (24-hr-old culture) and *B. subtilis* spores (4-day-old culture) within 4 hours at 37 C, but was devoid of killing effect within 240 hours at 25 C.

A7. *The Antibacterial Action of Flavonols.* JOSEPH NAGHSKI, MICHAEL J. COPLEY, AND JAMES F. COUCH, Eastern Regional Research Laboratory, Department of Agriculture, Bureau of Agricultural and Industrial Chemistry, Philadelphia 18, Pa.

During a recent study on the antagonistic action of flavonols to the antibacterial action of dicoumarol, it was observed that quercetin exerted some inhibition on *Staphylococcus aureus*. The antibacterial action of quercetin was most pronounced in the acid range, being negligible above pH 7. The degree of activity toward various organisms was determined in nutrient broth at pH 6.5 by measuring the turbidity of bacterial growth with a Klett-Sumerson photoelectric colorimeter. Quercetin in concentration of 0.075 to 0.10 mg per ml produced complete inhibition of *Staphylococcus aureus*, *Staphylococcus albus*, *Aerobacillus polymyxa*, and *Brucella abortus*; in concentration of 0.15 mg per ml only partial inhibition was obtained of a strain of a group D and a group E streptococcus, and the gram-negative organisms: *Escherichia coli*, *Salmonella oranienburg*, *Proteus* sp., *Pseudomonas aeruginosa*, *Pseudomonas angulata*, *Pseudomonas tobaci*, and *Aerobacter aerogenes*. Of several fungi investigated, *Mucor racemosus* was inhibited 25 to 30 per cent by 0.15 mg per ml, but *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium oxysporum* var. *lycopersici*, *Penicillium notatum*, and *Actinomyces fradii* were not affected. Owing to limited solubility, concentrations higher than 0.15 mg per ml were not feasible. Bactericidal action was demonstrated toward *S. aureus* in concentrations of 0.10 and 0.15 mg per ml and the rate of killing was proportional to the concentration. The activity of quercetin was lost in the presence of serum and iron but was not affected by cysteine. The glycosides of quercetin (rutin and quercetrin) were inactive.

A8. *Influence of Straw Mulching on Biological Activity and Soil Condition.* T. M. MCCALLA, U. S. Department of Agriculture, Soil Conservation Service, Research and Nebraska Agricultural Experiment Station, Lincoln, Nebr.

During the years 1944, 1945, and 1946 field plots, 14 by 20 feet, were operated at the Agronomy Farm, Lincoln, Nebraska. The plots were planted to corn and mulched with straw at the rates of 0, 2, 4, and 8 tons per acre. The straw was applied between the rows after the corn was planted, usually in late May or early June. Soil population, nitrates to a depth of 6 feet, aggregation, and stability of lumps to falling water drops were determined. Soil temperature



and yields were also measured. The straw mulch increased the number of fungi, actinomycetes, and bacteria. The increase in numbers corresponded with an increase in the amount of straw mulch applied, and occurred largely in the 0- to 1-inch soil depth. Almost all of the straw mulch on each treatment, except where the 8 tons were applied, decayed in 1 year. Soil temperature was lowered by mulching. At some times the aggregation and stability of lumps to falling water drops was increased by mulching in the 0- to 1-inch depth. At other times there was no difference. The nitrate content was slightly higher on the plots mulched with 4 and 8 tons of straw, but slightly lower on the plots with 2 tons of straw. This agrees with results previously reported for light mulches. Corn yields were increased by the use of the straw mulch. The highest occurred on the plots mulched with 8 tons of straw. No erosion by wind or water was observed on the mulched plots.

*A9. Decomposition of Wheat Straw by Soil Fungi.* ROY C. DAWSON, U. S. Department of Agriculture, Soil Conservation Service, Research and Nebraska Agricultural Experiment Station, Lincoln, Nebr.

Wheat straw is commonly used as a cover for the protection of land against soil and water losses. Decomposition takes place owing to the action of various microorganisms. Since the amount of vegetation is limited, the rate of decomposition is of vital importance. Ten-gram portions of air-dried, ground wheat straw were placed in 500-ml flasks, sterilized, and inoculated with pure cultures of various fungi. The moisture content was then adjusted and the flasks inoculated and incubated at 25 C for 7 days. Constant aeration and removal of carbon dioxide and ammonia were effected by slowly circulating a stream of air through the flasks. The carbon dioxide and ammonia removed were determined at daily intervals for the first 5 days and again on the 7th day. Loss in weight of the plant material was determined by analytical weighing, before and after decomposition. The oxidase reaction of the fungi was determined by streaking on tannic acid agar. The formation of a brown halo indicated the presence of oxidases. The rate of decomposition of wheat straw varied widely with the different fungi. Those producing oxidases on tannic acid agar were more active decomposers of wheat straw than those failing to produce oxidases. A high degree of correlation was found between carbon dioxide production and loss of weight during the decomposition process. Final reaction of the decomposed residue varied within the pH range 3 to 8.5, depending on the fungus involved. No relationship was found between final reaction and carbon dioxide production. Alfalfa straw decomposed more rapidly and liberated greater amounts of ammonia than wheat straw.

*A10. The Flora of Feces of Hens as Influenced by Various Carbohydrates in a Biotin-deficient Ration.* K. R. JOHANSSON, STANLEY K. SHAPIRO, AND W. B. SARLES, University of Wisconsin, Department of Agricultural Bacteriology, Madison 6, Wis.

Studies were made on the fecal flora of 24 White Leghorn hens separated into

groups of four, kept in wire-bottomed cages, and fed various rations. Group VI received a practical grain ration, and the other groups were fed a synthetic basal ration deficient in biotin, but with added carbohydrates as follows: group I, sucrose; group II, dextrin; group III, sucrose and lactose; group IV, sucrose and 200 micrograms of biotin per kilo; group V, sucrose and whey (weight of whey adjusted to furnish same per cent lactose as in group III). Fecal samples from group I were nearly devoid of coliform organisms; yeasts largely replaced the coliform flora in this group. Group III had the highest coliform count. Feces from groups II and V contained about 10 times the numbers of coliforms found in groups IV and VI. In all cases, total plate counts in tryptone yeast extract glucose agar were lower than thioglycolate dilution counts. Group IV gave the lowest count in the former medium, while the other groups were two to five times higher. Group II maintained the highest thioglycolate count; other groups averaged approximately one-half this number. The highest lactic count was found in group III and the lowest in group IV. In general, the fecal flora of groups II and V was more similar to that of group VI than to that of other groups. Simultaneous biochemical studies by Cravens and Couch suggest that there was intestinal synthesis of biotin in hens fed the basal ration plus dextrin (group II).

*A11. A Comparison of Rapid and Tube Antigens in Agglutination Tests on Poultry Blood.* DALE O. GALLOWAY AND D. FRANK HOLTMAN, University of Tennessee, Department of Bacteriology, Knoxville, Tenn.

Tests made on poultry blood with pullorum rapid antigens, T. G. and K formulae, and pullorum and fowl-typhoid tube antigens have shown considerable variability in the nature and the extent of reaction. Blood specimens, subjected to the rapid test, were classed as T. G. negative, K negative; T. G. negative, K unknown; T. G. negative, K positive; T. G. positive, K negative; T. G. positive, K unknown; T. G. positive, K positive. When these six classes were subjected to six different strains of pullorum, including two Canadian variants, and three different strains of fowl-typhoid tube antigens, marked variability in reaction was observed. A considerable number of the blood specimens positive to rapid antigen were negative to tube antigens. Some agglutinated only certain tube antigens, and were negative to others. Bloods agglutinating the K rapid antigen were more inclined to agglutinate tube antigens than those agglutinating the T. G. formula. It is conceivable that oversensitivity of rapid antigens might account for a greater number of positives than were attained with the tube antigens. Also, it is possible that dissociation of strains used in the preparation of tube antigen might result in fewer positives with such bacterial suspensions, even though the cultures employed had been recently secured from reliable sources and were assumed to be in their normal antigenic phases.

*A12. The Fermentation of Pectin and Pectic Acid by Clostridium felsineum Strain No. 195.* LOUISE F. POTTER AND ELIZABETH MCCOY, University of Wisconsin, Department of Agricultural Bacteriology, Madison 6, Wis.

*Clostridium felsineum* rapidly ferments pectin and pectic acid in 2 per cent suspensions. The base medium used in these studies consists of aqueous liver extract (equivalent to 2 per cent liver), 0.05 per cent each of  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $(\text{NH}_4)_2\text{SO}_4$  and a trace of  $\text{FeSO}_4$ . The pectin (2 per cent) is suspended in the base medium by blending in a Waring "blendor." In order to suspend pectic acid (2 per cent) it is necessary to adjust the pH of the medium to 5.0 to 6.0 while blending. The media are sterilized by autoclaving and the pH adjusted aseptically to 7.0 before inoculation. One-hundred-ml quantities of the media in 6-ounce bottles are inoculated with 10 ml of an 18- to 20-hour culture of *Clostridium felsineum*. The flasks are incubated at 37 C. At various lengths of time, analyses are made on the contents of the flasks. The pH, titratable acidity, and saponification values are determined as well as the amount of calcium pectate, total galacturonic acid, and free galacturonic acid. There is little difference in the rate of fermentation of pectin and pectic acid. Both are fermented rapidly, more than 80 per cent being fermented within 24 hours.

A13. *The Effect of Legume Hemoprotein on Nitrogen Fixation in Vitro.* H. F. NISS, HELEN A. MACHATA, AND P. W. WILSON, University of Wisconsin, Department of Agricultural Bacteriology, Madison, Wis.

Virtanen and Laine claim to have obtained fixation by *Rhizobium* sp. apart from the host plant when extracts of nodules containing hemoprotein pigment were supplied. Using more purified preparations, they were unable to secure fixation. Several species of *Rhizobium* and aqueous extracts (largely nodule hemoprotein) of soybean and pea nodules were added to a nitrogen-free medium. After incubation, the samples were analyzed for total nitrogen by a semimicro-Kjeldahl method. Variations in the procedure included incubation of the cells in the medium before the addition of the extract; preparation of the extract in the cold; extraction under carbon monoxide; filtering the crude extract through cheesecloth instead of a Berkefeld N filter. Some organic acids reported as intermediates or as stimulating fixation, such as oxalacetic, citric, or  $\alpha$ -ketoglutaric, were tested. A statistical analysis of the data from the macro experiments showed that no significant amount of nitrogen was fixed. The results are being checked through use of an isotopic technique in which  $\text{N}^{15}$  is used to test for fixation of minute quantities of nitrogen not detectable by the Kjeldahl method.

A14. *Effect of Soil Microorganisms on the Persistence of Plant Growth Regulators in Soil.* ARTHUR S. NEWMAN AND A. G. NORMAN, Camp Detrick, Frederick, Md.

The disappearance of 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, 2-methyl-4-chlorophenoxyacetic acid, and isopropyl N-phenyl carbamate from soils which have not been subjected to leaching is due to their decomposition by soil microorganisms. The compounds persist much longer in soils which have been autoclaved than in normal soils and disappear sooner from soils of high microbial activity than from soils of normal microbial activity. Also these plant growth regulators persist longer in soils of low moisture content

than in soils of optimum moisture content. The effect of these plant growth regulators on nitrification and plate counts of soil microorganisms is also discussed.

A15. *Thermal Death Rates of Yeast Cells.* HOWARD REYNOLDS AND SARAH B. DUNCAN, Bureau of Human Nutrition and Home Economics, U. S. Department of Agriculture, Washington, D. C.

Thermal death rates of yeast cell suspensions in buffered media (pH 4.7) have been studied to provide data for establishing thermal death time curves. Yeast cell suspensions were subjected to lethal heat and samples removed at intervals for enumeration of survivors. The logarithms of survivor counts tended to be linearly related to heating time when the data from several rate determinations were combined. Variance analysis of data from single rate determinations frequently indicated significant deviations from linearity. Uncritical interpretation of the latter would lead to the conclusion that the observed death rates were not logarithmic. More complete data indicate, however, that such apparent deviations from linearity can be attributed, for the most part, to exaggerated estimates of the precision with which the destruction caused by specified exposures to heat can be estimated. The low estimates of error variance obtained when data from single death rate determinations were analyzed caused relatively minor deviations from linearity to appear significant. When data from duplicate runs were combined, larger, and probably truer, estimates of error variance resulted. When examined in this manner, deviations from linearity were no longer significant and the results were not contradictory to the conclusion that the observed death rates were logarithmic. Computation of regression coefficients and standard errors of regression from grouped data yielded the best estimates of death rates at specified temperatures and permitted the setting of confidence limits to the death rates so determined. Points representing 99.99 per cent destruction taken from three or more thermal death rate curves provided data for constructing thermal death time curves.

A16. *Riboflavin Production by Ashbya gossypii.* FRED W. TANNER, JR., AND JAMES M. VAN LANEN, Northern Regional Research Laboratory, Fermentation Division, Peoria, Ill.

Since *Ashbya gossypii* is capable of synthesizing riboflavin in unusually large amounts under certain conditions, the development of conditions suitable for commercial production of the vitamin has been undertaken. The influence of size and age of inoculum, inoculum development procedures, sterilization techniques, incubation temperatures, and constituents of media have been studied. Best yields of the vitamin are obtained with small young inocula developed by transferring young (24 to 48 hours) stock slant cultures to liquid media and incubating at 28 C on a mechanical shaker for 24 hours. Inocula of 0.5 to 1.0 per cent by volume are used. Though there are no apparent effects upon growth, deleterious effects on riboflavin production are observed when media are autoclaved longer than 30 minutes at 15 pounds' pressure. The optimum tempera-

ture for riboflavin production is 28 C even though abundant growth is obtained over rather wide temperature limits. On a medium composed of 4 per cent glucose, 0.5 per cent peptone, and 0.5 per cent corn steep liquor solids, yields of 1500 to 600 micrograms per milliliter are obtained when optimum cultural conditions are used. When either corn steep liquor or peptone is omitted, riboflavin synthesis is decreased. Although animal stick liquor can replace peptone with nearly equivalent yields, use of proteinaceous products of plant origin only is less satisfactory.

*A17. Production of p-Cresol and Phenol from Tyrosine by Marine Mud Cultures.*

R. W. STONE AND H. E. MACHAMER, The Pennsylvania State College, Department of Bacteriology, State College, Pa.

Enrichment cultures of marine muds on a tyrosine sea water broth have produced either phenol or *p*-cresol from tyrosine but generally not both compounds. Three pure cultures were isolated from one *p*-cresol-producing mixed culture that grew actively under anaerobic conditions using *l*-tyrosine as a chief energy source. The organisms were small, nonpigmented gram-negative rods, sometimes slightly curved or bent in appearance, having the ability to reduce sulfate in sea water with the production of hydrogen sulfide. Two of the organisms produced phenol in yields of from 50 to 60 per cent of theoretical amount and the third produced *p*-cresol. The fermentations were conducted by two methods: in liter flasks of broth and in 2-liter flasks filled with crushed rock to increase the surface. The same products were found in both methods, but the presence of crushed rock increased the speed of fermentation from two to three times. Phenol was identified as the bromo-derivative and the aryloxyacetic-acid-derivative. *p*-Cresol was identified as the aryloxyacetic-acid-derivative only. Melting points and mixed melting points of these preparations from the crude neutral ether extract of fermentation liquors checked within 0.5 C with the corresponding known compounds, indicating that the individual organisms specifically produced either one compound or the other and not mixtures of the two.

*A18. A Comparison of Rates of Fermentation by Lactobacillus casei in Synthetic and Yeast Extract Media.* H. O. HALVORSON AND MARY R. MUEDEKING, University of Minnesota, Department of Bacteriology and Immunology, Minneapolis, Minn., and University of Kentucky, Department of Bacteriology, Lexington, Ky.

The rate of fermentation of glucose by *Lactobacillus casei* depends on concentration of nutrients. In a calcium-carbonate-buffered yeast extract medium, the fermentation rate was a function of the yeast extract concentration in the range 10 to 40 per cent. In a synthetic medium, the rate was much lower. The effect of yeast extract was shown not to be due to enrichment with nitrogen. The rate of fermentation in a synthetic medium was not markedly influenced by concentrations of known B vitamins. The initial rates were nearly equal in media containing concentrations in the ratio 1:2:4. As cultures aged, that with the highest concentration exceeded the others slightly. Cells in calcium-carbonate-

buffered yeast extract medium fermented glucose in concentrations as high as 12 per cent to completion, the viable count decreasing only slightly below the maximum. In a similar time cells in a synthetic medium had not completely fermented 2 per cent glucose. The pH is a possible limiting factor in synthetic media. After fermentation ceased in a riboflavin-deficient, synthetic medium, the addition of pure riboflavin had no effect on fermentation or growth. Other B vitamins singly, a mixture of B vitamins, and a mixture of purines and pyrimidines had no effect.

*A19. The Effect of Added Nitrogen on the Formation of Protein and Fermentation by Yeast.* S. L. ADAMS, J. K. WOODS, AND W. H. STARK, Joseph E. Seagram & Sons, Inc., Research Department, Louisville, Ky.

A study was made to determine the feasibility of increasing the protein content of grain fermentation by-products by the addition of aqueous ammonia, ammonium salts, and urea. These substances were added to a standard mash, composed of 92 per cent corn and 8 per cent barley malt, immediately before inoculation with a distillery type yeast. On the basis of 51 paired fermentations, the yield of alcohol in the presence of urea equivalent to 0.4 g nitrogen per liter was 5.79 proof gallons per bushel while the control was 5.89 proof gallons per bushel. In no case was the yield of alcohol with added nitrogen greater than the control. This decrease in alcohol yield was not caused by differences in starch conversion or completeness of fermentation since the concentration of residual sugar, after acid hydrolysis, was always less in the presence of added nitrogen than in the control. The disparity between the alcohol yield in the control fermentations and fermentations containing added ammonium sulfate or aqueous ammonia was considerably greater than in the case of urea. When urea was added to the extent of 0.36 g nitrogen per liter, over 90 per cent was converted to protein. By the addition of urea or ammonium sulfate the protein content of fermentation by-product grains can be increased; this increase however is accomplished at the expense of alcohol production.

*A20. Riboflavin Synthesis by Eremothecium ashbyii in Synthetic and Natural Substrates.* HELEN NORRIS MOORE AND G. DE BECZE, Schenley Distilleries, Inc., Biochemical Research Laboratory, Lawrenceburg, Ind.

Riboflavin synthesis by *Eremothecium ashbyii* has been studied in synthetic media and distillers' stillage. Maintenance of activity in stock cultures, preparation of active inocula, and propagation of the organism have been investigated by the shaken flask and aerated submerged culture technique. Cultures stored at 3 to 10 C and subcultures of lyophilized organisms were observed to lose their capacity for synthesizing riboflavin. In the latter case, the change was irreversible; but with the former, the change was gradual and could be restored by suitable procedures. At room temperatures, however, activity was maintained for 6 months in semisolid maltose peptone medium without transfers. Activity was not lost in transfers, provided only brilliant orange colonies picked from streak plates were propagated in alternating substrates of varying composition. The

organism grew profusely in agar media at pH 5.5 to 6.8 and subcultures of such growth were active after 12 transfers in broth. At a pH of 4.5 growth was scanty, and the second transfer into broth at that pH failed to grow. Cultures of known activity, when propagated at a pH 5.5 to 6.8 for 72 hours at 28 C, synthesized riboflavin up to 2,300  $\mu\text{g}$  per g solids in Czapek's sucrose broth; 4,000 in yeast extract peptone glucose broth; 4,600 in stillage; and 20,000 in properly supplemented stillage.

**A21. Factors Affecting the Production of Citric Acid in Submerged Cultures.** E. O. KAROW, Merck and Co., Inc., Research and Development Department, Rahway, N. J.

A number of fungi are capable of producing citric acid in submerged culture. Yields of 50 to 65 per cent, as anhydrous citric acid, of the sugar consumed have been obtained with a strain of *Aspergillus wentii* isolated from the soil. Factors influencing the production of citric acid are the strain of the organism, balance of mineral constituents of the medium, control of pH, and highly aerobic conditions. The replacement culture technique was used. Experiments were carried out in 250-ml flasks on a shaking machine. A medium consisting of 150 g sucrose; 1.0 g urea; 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.08 g  $\text{KH}_2\text{PO}_4$ ; 0.15 g KCl; 0.02 g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ; and 0.01 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  in 1 liter distilled  $\text{H}_2\text{O}$  adjusted to pH 2.0 with HCl, supported good growth of the organism. Preformed cell material, developed in this medium, readily produced citric acid in a medium containing 158 g sucrose; 0.5 g urea; 0.05 g  $\text{KH}_2\text{PO}_4$ ; 0.15 g KCl; 0.02 g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ; and 0.01 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  in 1 liter distilled  $\text{H}_2\text{O}$  adjusted to pH 2.0 with HCl.

**A22. Actidione, an Antibiotic Produced by *Streptomyces griseus*.** ALMA J. WHIFFEN, The Upjohn Company, Antibiotics Research Department, Kalamazoo, Mich.

Strains of *Streptomyces griseus* which produce streptomycin also yield an antibiotic, actidione, which differs from streptomycin in being ether-soluble, inactive against bacteria, and highly active against yeasts. The discussion of this antibiotic is divided into three parts: (1) the production of actidione, the influence of the culture medium and the strain of *S. griseus* upon the yield of actidione; (2) the method of assay, its accuracy, and the importance of inoculum-size effect; and (3) the antibiotic activity of actidione, the antibiotic spectrum, and the development of resistance to actidione.

**A23. A Bacteriophage in the Streptomycin Fermentation.** E. C. SAUDEK AND D. R. COLINGSWORTH, The Upjohn Company, Antibiotics Research Department, Kalamazoo, Mich.

The presence of a lytic agent capable of lysing cells of *Streptomyces griseus* has been detected in cultures and streptomycin fermentation beers. The phage will pass through a bacterial filter, and may be propagated in the presence of young growing cultures of *S. griseus*. Cultures both resistant and sensitive to the phage have been isolated and cultivated. When added to a fermentation seeded with

the phage-sensitive strain of *S. griseus*, the phage will partially or completely prevent streptomycin production. Quantitative procedure for measuring the amount of phage in samples consisted of the plaque method using the phage-sensitive strain of *S. griseus*. The test method indicated millions of phage particles present in seed and production fermentations where poor or thin growth was obtained with little or no streptomycin production. Electron micrographs show this agent to be similar in size and shape to some of the better known bacteriophages.

*A24. The Production of Streptomycin in Stationary Culture on Liquid and Solid Substrates.* H. B. WOODRUFF, Merck & Co., Inc., Research and Development Department, Rahway, N. J.

Various factors which influence the production of streptomycin in surface culture, on a basal medium of nutrient broth with 1 per cent glucose, have been investigated. Improvement in yield was obtained by altering the medium to contain 1 per cent NaCl, by substituting Difco tryptose or certain samples of yeast extract for Difco peptone, and by incorporating Fe and Zn salts in the medium. The addition of  $Zn^{++}$  gave rise to rapid pellicle formation but streptomycin formation was inhibited. The addition of  $Fe^{++}$  had a converse effect; streptomycin production was enhanced but pellicles had about 50 per cent of normal weight. With a medium containing distilled water, an optimum balance of 30 mg of  $FeSO_4 \cdot 7H_2O$  and 10 mg  $ZnSO_4 \cdot 7H_2O$  per liter was found, but it was necessary to vary the ratio to obtain maximum activity under production conditions with waters from various sources. Inoculum for surface cultures was developed satisfactorily by growth of *Actinomyces griseus* on moist rice bran. Adaptation of the stock culture to grow rapidly on rice bran increased the number of spores formed. Streptomycin was produced in the rice bran medium during growth of *Actinomyces griseus*. A maximum of 1,400 micrograms of streptomycin per gram of substrate was recovered by elution from the bran with acid. Highest yields were obtained from samples of rice bran supplemented with inorganic salts.

*A25. An Oval Tube Method for the Determination of the Bactericidal Effectiveness of Various Sterilizing Agents.* FRANKLIN W. BARBER, ROBERT P. MYERS, AND EDNA K. HARRIS, National Dairy Research Laboratories, Inc., Baltimore 17, Md.

The oval tube method for testing various sterilizing agents is the determination, under controlled conditions, of the time required to obtain 99.9 per cent destruction of the test organism by a known concentration of the sterilizing agent. A germicidal solution of known concentration is inoculated with a suspension of the test organism in distilled water. Subtransfers, at specific time intervals, are made with a calibrated loop into oval tubes of melted agar. Controls to detect resistant contaminants in the test solution and any bacteriostatic action against the test organism by carry-over of the germicide from the test solution are made for each test, in addition to a colony count on the inoculum used. All tubes are slanted and after incubation are counted. The time required to obtain 99.9



per cent destruction of the organism is then calculated. The method has merit in that the determination of 99.9 per cent destruction of test organism is more easily reproducible than the determination of complete destruction; an accurate evaluation of the sterilizing agent for specific organism under specific conditions is possible; the method is easily applicable to many test organisms and sterilizing agents; and a minimum of equipment, material, and incubator space is required for a large number of tests. The oval tube method has been used to determine satisfactorily the bactericidal effectiveness of hypochlorites and a number of the quaternary ammonium compounds for cultures of *Staphylococcus aureus* (FDA no. 209), *Escherichia coli*, and species of *Micrococcus*, *Microbacterium*, and *Pseudomonas*.

*A26. Difficulties Involved in the Evaluation of Quaternary Ammonium Salts.*

ROBERT A. QUISNO, MILTON J. FOTER, AND HARRY L. RUBENKOEING,  
Research Laboratories of The Wm. S. Merrell Company, Department of  
Bacteriology, Cincinnati, Ohio.

The usual phenol coefficient test is inadequate for the evaluation of germicides other than the phenolic types. A discussion of test procedures is presented in order to assist in understanding the difficulties of properly evaluating quaternary ammonium salts. Data are given to show that germicidal activity varies with the procedure employed and that inconsistencies are not limited to surface tension and ionic attraction effects. Compounds which are incompatible with quaternary ammonium salts are discussed. The pitfalls and errors found in testing these compounds are shown by several different test methods. It is pointed out that no one test method is suitable for all germicides, or applicable to all uses of a given germicide. Choice of a germicide must be based on its effectiveness under the conditions of use, and test methods should be devised to simulate these conditions as closely as possible.

*A27. Physical Action of Surface-active Cations upon Bacteria.* E. W. KIVELA,  
W. L. MALLMANN, AND E. S. CHURCHILL, Michigan State College, De-  
partment of Bacteria and Public Health, East Lansing, Mich.

Reversal of surface-active cations can be reversed both by centrifugal washing and by simple dilution accompanied by vigorous shaking without the use of a neutralizing agent. This was shown both by bacteriological methods and also by physical methods using electrophoretic mobilities as a criterion of the amount of cation absorbed upon the surface of an organism. Using a *Bacillus subtilis* spore suspension, 50 to 70 per cent of the spores could be recovered as tested bacteriologically. Physically, the positive charge produced by the cation was removed and the normal charge of the spore restored. With vegetative cells such as *Escherichia coli* and *Eberthella typhosa* the normal electrical charge could be restored, but the organisms could not be revived. When tested by standard techniques, no reversal was obtained. High dilutions of cation above germicidal activity will cause clumping of bacteria as observed microscopically. This is evidently due to the proper concentration which neutralizes the charge of

the bacteria and allows them to agglomerate. Concentrations such as used for germicidal purposes place a sufficient charge upon the bacteria effectively to disperse them. As tested by freezing-point depression methods, the osmotic pressure of the cation was found to be quite marked. Since their concentration upon the bacterial cell is greater than the concentration in solution, this osmotic effect may be very important in the mode of action of this type of compound.

*A28. Inhibitors for Neutralizing the Germicidal Action of Quaternary Ammonium Compounds.* GEORGE R. WEBER AND LUTHER A. BLACK, U. S. Public Health Service, Water and Sanitation Investigations, Cincinnati, Ohio.

In making swab-rinse plate counts of food utensils sanitized by quaternary ammonium compounds, it has been suggested that an inhibitor be used to arrest germicidal action, similar to neutralization of chlorine by sodium thiosulfate. Such an inhibitor is also necessary in certain investigations of cationic germicides. A gross screening procedure was first used to evaluate compounds. Phosphate buffer, culture (*Escherichia coli*), and inhibitor were mixed in a petri dish, allowed to act for 10 minutes, following which germicide was added. Ten minutes later the petri dish was poured with concentrated agar known to arrest germicidal action. Each compound exhibiting satisfactory inhibitory action was further investigated by placing in a test tube appropriate amounts of phosphate buffer, culture, and germicide, followed by periodic plating. After a definite killing curve was established for each germicide, experiments were repeated introducing adequate amounts of inhibitors into the germicidal solution after 2½ and 5 minutes' exposure. By both procedures duponol WA, lecithin in tween 80, naphuride sodium, tergitol WA 7, and triton X 200 are effective inhibitors for alkyl-dimethylbenzyl-ammonium chloride. Studies utilizing duponol WA similarly inhibited dimethyloctadecenylethyl-ammonium bromide, alkenyldimethylethyl-ammonium bromide, alkylarylpyridinium chloride, and cetylpyridinium chloride. Further studies are under way utilizing other compounds.

*A29. Some Studies on Quaternary Ammonium Compounds.* R. R. RUCKER AND ERLING J. ORDAL, U. S. Fish and Wildlife Service and Department of Microbiology, University of Washington, Seattle, Wash.

Certain quaternary ammonium compounds, when used under carefully controlled conditions, are effective in controlling gill disease in salmonid fishes. There is, however, a relatively narrow margin of safety between the concentrations effective in controlling the disease and the concentrations toxic to fish. In this study the toxicity to fish of various members of the homologous series of alkyl-dimethylbenzyl-ammonium chlorides has been compared with their lethal effect against *Chondrococcus columnaris*, a myxobacterium causing disease in fish. The dodecyl and tetradecyl homologues were found to be relatively more effective when the lethal action against bacteria was compared with toxicity to fish. It was found further that the germicidal action of some members of the homologous series was enhanced by the presence of organic material containing nitrogen.

A30. *The Fungicidal Effects of the Fatty Acids on Species of Trichophyton.*

EMANUEL GRUNBERG, Hoffman-La Roche, Inc., Department of Chemotherapy, Nutley 10, N. J.

The fungicidal action of the series of fatty acids (formic acid to undecylenic acid) at various pH levels was investigated. It was observed that fungicidal action within 24 hours was present only at pH 5.5. This activity increased with the length of the carbon chain, following Traube's rule. Four strains of *Trichophyton gypsum* and four strains of *Trichophyton purpureum* were employed. Although it has been claimed that there is a difference in resistance to fungicides between the two species, none was apparent in *in vitro* tests with the fatty acids. The accuracy of various methods for testing fungicides is discussed, and a modified form of Emmon's test, which eliminates as many variables as possible, is suggested. A consideration of the results seems to indicate that the fungicidal activity of the fatty acids can be correlated with the undissociated fraction. Experiments were carried out to determine the relative irritant effects of the fatty acids.

A31. *The Deterioration of Photographic Negatives by Microorganisms.* JOHN A.

JUMP AND W. G. HUTCHINSON, University of Notre Dame, Notre Dame, Ind., and University of Pennsylvania, Philadelphia, Pa.

Photographic negatives are subject to damage by microorganisms when stored under conditions of high humidity in natural and simulated tropical environments. Certain fungi may cause the appearance of colored spots on a negative by oxidizing the dye used in the antihalation backing. This dye is present in the leuco form following its reduction during processing. Several species of *Penicillium* are particularly active in this respect. This type of spotting appears on prints made from such negatives and is especially troublesome when it occurs in low density areas. Partial liquefaction of the emulsion by bacterial action and etching of the negative surface by fungus mycelium may occur in tropical chamber tests. A suitable agent for the prevention of this type of deterioration was found in a mixture of high molecular weight alkyldimethylbenzyl-ammonium chlorides marketed as roccal. This combined the essential properties of non-toxicity to personnel and simplicity of application. No undesirable changes in the physical or chemical characteristics of the negative were brought about by this treatment. Negatives were dipped after processing and washing in a 10 per cent solution of commercial roccal (10 per cent active ingredients). These negatives withstood testing in a tropical chamber for 6 months where they were subjected to leaching from condensation, high humidities, and a heavy inoculum of microorganisms. Control negatives under the same conditions began to show fungus growth in 2 days and were completely worthless in 2 weeks.

A32. *The Fouling of Optical Glass by Microorganisms.* W. G. HUTCHINSON,

University of Pennsylvania, Department of Botany, Philadelphia, Pa.

The fouling of glass surfaces in optical instruments in use or in storage in the

humid tropics may be brought about by molds and bacteria. The presence of a mass of mold and bacteria on a surface which is continually in focus, such as a reticle, will greatly obscure vision. In addition, on some types of glass deep etching of the surface may occur along the path of mycelial growth, which may render a prism or lens unfit for use. These microorganisms secure nutrition from organic detritus upon the glass surfaces or may spread from cork pads used to support prisms and lenses. Of most frequent occurrence are several species of *Penicillium* and *Aspergillus*, and also *Monilia crassa*, *Sporotrichum* sp., *Stachybotrys atra*, and *Bacillus* sp. Control may be secured by the hermetical sealing of instruments or by the use of a volatile fungicide within the instruments. Such a fungicide must be selected with care to avoid metallic corrosion, sublimation or condensation on glass surfaces, softening of cement, and any toxic effect for personnel. In tests conducted in the humid tropics, *meta*-cresyl acetate (cresatin) has given complete protection of binoculars for over 2 years while unprotected instruments have become badly infested in 3 weeks.

*A33. Relations of Sulfate-reducing Bacteria to Corrosion of Steel in Sea Water.*

ROBERT L. STARKEY AND JOHN D. SCHENONE, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.

Corrosion products on steel exposed in the ocean for several weeks or longer were found to consist of a surface layer of red ferric hydrate and an underlying layer of black graphitelike or crusted material that contained iron sulfide. Similar black sulfide-containing corrosion products accumulated beneath the fouling organisms that became attached to the specimens. Sulfate-reducing bacteria were abundant in the products, particularly in the black products. Their numbers, determined by a culture method that probably indicated considerably less than the actual numbers, were found to be from a few thousand to more than one billion per gram. These bacteria are presumed to have produced most of the sulfide found in the corrosion products. The surface layer of corrosion products and fouling organisms that forms on steel surfaces exposed in sea water serves to remove oxygen from the water as it diffuses to the metal surface, thus favoring development of the anaerobic sulfate-reducing bacteria within the range tolerated by these bacteria. The black corrosion products had low redox potentials indicative of reducing conditions. The sulfate-reducing bacteria are presumed to play the same role in the anaerobic corrosion of steel in sea water that they do in soils. The bacteria were found in abundance in marine sediments that contained iron sulfide. They were also encountered in coastal waters as well as in fouling detritus. The latter may serve to distribute the bacteria throughout the ocean.

*A34. The Bacteriology of Poultry Evisceration.* MILLARD F. GUNDERSON AND KENNETH D. ROSE, University of Nebraska, College of Medicine, Department of Pathology and Bacteriology, Omaha, Nebr.

Wartime investigation of the process of poultry evisceration was instituted by the Quartermaster Corps with a view toward improving the resultant product for

use by our armed forces. To facilitate this work, spot plates were used to judge the bacteriological condition of the poultry. A grading system was devised to permit the bacteriological evaluation of such poultry and of the plant processes. It was found that the method of preparation and the environment imposed a transient flora on the skin surfaces of poultry in addition to the normal flora already present thereon. This transient flora was of intestinal origin and assumed an important place in the categories of bacteria present. The process of scalding and other operations, as practiced, facilitated this gross contamination. *Salmonellas* were isolated from the skin surfaces of a small percentage of the birds. Using certain precautions and innovations in processing, birds can be eviscerated under factory conditions with a relatively low bacteria count as evaluated by the grading method. This is true particularly with reference to the bacteria originating from contamination by intestinal contents.

*A35. Some Bacteriological Aspects of the Commercial Production of Boned Chicken.*

KENNETH D. ROSE AND MILLARD F. GUNDERSON, University of Nebraska, College of Medicine, Department of Pathology and Bacteriology, Omaha, Nebr.

Employing techniques outlined by the Committee on the Microbial Analysis of Foods of the American Public Health Association, analyses were made for total and coliform bacterial numbers, for hemolytic *Staphylococcus aureus*, and for members of the *Shigella* and *Salmonella* group at various stages of boned chicken production and in the final chicken-containing products. Cooking of eviscerated chicken prior to boning reduces the bacterial load to as low as 73 organisms per gram of meat. Cooked meat coming into contact with the hands of workers and contaminated equipment becomes seeded with a heavy inoculum of bacteria which rapidly increases to as many as 9 millions of bacteria per gram during the approximately 2½ hours required for the complete boning procedure. Further increases in bacterial numbers result from plant production malpractices, the most important of which is a further holding time of 1 to 3 hours at room temperature before freezing. In a chicken-containing frozen food, members of the genus *Salmonella* are rapidly killed by storage at -14 F, but from 2 per cent to 20 per cent of the initial inoculum can survive storage for 92 days. The normal flora is killed more slowly. The bacterial count in cold-stored chicken-containing food depends primarily on the initial contamination rather than on the storage time, as is demonstrated by excessive contamination in material examined after 3½ to 5½ months' storage. Hemolytic *Staphylococcus aureus* but not members of the *Shigella* and *Salmonella* group have been isolated from frozen food.

*A36. An Outbreak of a Bacterial Disease of Lobsters.* S. F. SNIESZKO AND C. C. TAYLOR, U. S. Fish and Wildlife Service, Department of the Interior, Kearneysville, W. Va., and Boothbay Harbor, Maine.

During the summer of 1946 an outbreak of a new disease caused considerable losses among lobsters kept in pounds along the coast of Maine. The diseased lobsters had a pink discoloration of the ventral side of the abdomen, the blood

was pink also, less viscid, with a much prolonged clotting time and reduced number of corpuscles. Large numbers of micrococci of the *Gaffkya* type in pure culture frequently were present in the blood of the diseased lobsters. This organism was readily isolated from the diseased lobsters and was grown on the standard laboratory media. In the blood of the infected lobsters the organism was present in tetrads surrounded with a thick pseudocapsule. Lobsters inoculated intramuscularly and intravenously with the pure cultures of this organism died within 2 weeks with the typical symptoms. From the blood of these lobsters the organism was reisolated in pure culture. In random experiments, the lobsters inoculated by injection died, even if fed a diet containing sulfonamides. Lobsters fed infected lobster meat or the microorganism in pure culture did not contract the disease. Diet containing sulfonamides reduced the mortality of those lobsters which had spontaneously contracted the disease.

A37. *A Study of a Microorganism Causing a Bacterial Disease of Lobsters.* E. R. HITCHNER AND S. F. SNIESZKO, University of Maine, Orono, Maine, and Fish and Wildlife Service, Kearneysville, W. Va.

Since the bacteria which caused the disease of lobsters reported by Snieszko and Taylor grew sparingly on beef extract peptone base media, a yeast water, trypticase base medium was used in most of the studies. On artificial media, the organisms occur as gram-positive, nonmotile, noncapsulated cocci (0.8 to 1.1 microns) in typical tetrad arrangement, but are capsulated in lobster blood. Colonies are grayish white, circular, raised, 1 to 2 millimeters in diameter; on agar slants the growth was scant, beaded (discrete colonies); in liquid media growth was scant with granular sediment; facultative as to oxygen. Acid, but no gas, is produced from glucose, lactose, sucrose, maltose, raffinose, mannitol, glycerol, salicin; dulcitol was not fermented; nitrates were not reduced; gelatin was not liquefied; starch was not hydrolyzed; indole, hydrogen sulfide, and acetyl-methyl-carbinol were not produced; urea was not hydrolyzed; ammonium salts were not utilized as a sole nitrogen source; no growth occurred on potato; slight acid reaction was produced in litmus milk; beta hemolysis was produced on human blood agar. Growth temperature ranged from 6 C to 44 C; optimum 30 to 35 C. The organism is pathogenic for lobster. A comparison of the organism with two strains of *Gaffkya tetragena* shows little similarity in characteristics. It would seem that the organism differs sufficiently from previously described species of the genus *Gaffkya* as to warrant tentatively a new species designation. *Gaffkya homari* (from *Homarus americanus*—the lobster) is suggested.

A38. *Occurrence of Multiple Salmonella Types in Spray-dried Whole Egg Powder.*

MATHILDE SOLOWEY AND AARON ROSENSTADT (1) Microbiology Research Division, Bureau of Agricultural & Industrial Chemistry, U. S. Department of Agriculture, Philadelphia, Pa., AND EARLE H. SPAULDING and CECILIA CHERMERDA (2) Department of Bacteriology & Immunology, Temple University School of Medicine, Philadelphia, Pa.

The possible presence of multiple *Salmonella* types was investigated in 171

samples of high (4 to 6 per cent) moisture egg powder from which 2 to 30 colony isolates were serologically identified. Single *Salmonella* type contamination was observed in 72 of 86 samples from each of which two presumptive positive colony isolates were identified, in 16 of 21 samples from each of which 3 to 10 colony isolates were identified, and in 22 of 64 samples from each of which 20 to 30 colony isolates were identified. The remaining 61 of the 171 samples of powder were contaminated with 2 to 6 *Salmonella* types, 33 with 2 types, 13 with 3 types, 10 with 4 types, 3 with 5 types, 2 with 6 types. Of samples yielding a single *Salmonella* type, regardless of the number of colony isolates identified, *Salmonella oranienburg* or *Salmonella montevideo* were most frequently isolated. When occurring in combinations with other types, they made up the largest number of identified colonies. No combinations occurred more frequently than any other, although certain types appeared in combinations oftener than others, i.e., *S. oranienburg*, *S. tennessee*, *S. montevideo*, *S. anatum*, *S. bareilly*. In general, the probability of isolating more than one *Salmonella* type per sample of powder is increased as the number of presumptive positive colony isolates identified per sample is increased.

*A39. Growth and Survival of an Experimentally Inoculated Staphylococcus aureus in Frozen Precooked Foods.* A. W. PHILLIPS, JR., AND B. E. PROCTOR, Massachusetts Institute of Technology, Department of Food Technology, Cambridge, Mass.

A variety of commercial frozen precooked meat, poultry, and fish products have been examined for their ability to support the growth and survival of an experimentally inoculated culture of enterotoxic *Staphylococcus aureus*. Food samples were inoculated with water suspensions of *S. aureus* and stored at  $-18^{\circ}\text{C}$ . The numbers of *S. aureus* surviving after 9 months were determined. A similar group of inoculated food samples was employed for the estimation of the growth of *S. aureus* between 5 and  $37^{\circ}\text{C}$ . Plate counts were made on Chapman's NaCl agar after 48-hour incubation at  $37^{\circ}\text{C}$ . Results indicate creamed food products, such as chicken a la king, ham a la king, creamed tuna, creamed salmon, are good substrates for *S. aureus*. Samples of creamed food products heavily inoculated with *S. aureus* ( $10^5$  cells per g of food) were found to contain large numbers of these organisms after storage at  $-18^{\circ}\text{C}$  for 9 months, although there were some reductions in *S. aureus* counts.

*A40. The Control of Dust-borne Organisms by the Impregnation of Textiles with a Germicidal Oil-Water Emulsion.* I. L. SHECHMEISTER AND FRANCIS S. GREENSPAN, University of California, Department of Bacteriology, Berkeley, Calif., and The New York Hospital, New York, N. Y.

The study carried on under the auspices of the U. S. Navy, dealt with the evaluation of (1) the germicidal nature of oil-water-roccal emulsion, and (2) the effective duration of a single impregnation of blankets with this emulsion. The germicidal emulsifiable oils consisted of a highly refined mineral oil, a single non-ionic emulsifying agent (tween 81), and a quaternary ammonium compound (roccal). A method was developed for quantitative impregnation of the textiles

in a commercial laundry by adding the desired amount of emulsifiable oil and roccal to the last water rinse. This impregnation process was effectively carried out with either cotton or woolen textiles at 85 to 125 F, and at pH 4.0 to 10.5. Roccal not only rendered the nonionic emulsion cationic, but was also responsible for the germicidal nature of this preparation. Four similar groups of blankets were treated respectively with (1) emulsifiable oil, (2) roccal, (3) both oil and roccal, and (4) neither oil nor roccal. These blankets were used by men living in the same room, and were regularly sampled for their bacterial content. Impregnation with both oil and roccal resulted in much greater reduction of recoverable blanket bacteria, as compared with the use of either agent alone. Under the conditions of the experiment, the germicidal nature of the oil-roccal emulsion imparted a self-sterilizing property to the blankets. In another experiment, monthly sampling of similarly used treated (with oil-roccal emulsion) and control blankets revealed a reduction of over 90 per cent in the number of organisms isolated from the treated as compared to the untreated blankets. The effect of a single treatment lasted for at least six months.

A41. *A Cellulose-decomposing Bacterium in Sludge.* R. E. HUNGATE, Washington State College, Department of Bacteriology and Public Health, Pullman, Wash.

A gram-negative anaerobic rod which is extremely active in cellulose decomposition has been isolated from sludge. Strains of the organism appear to be a constant component of sludge, though never present in large numbers. The organism grows readily on sugars and in sugar media and exhibits great variation in size of the cells, with many cells unevenly stained. Reducing materials accumulate in old cultures. Volatile acid and hydrogen are among the fermentation products.

A42. *The Egg Injection Method in the Evaluation of Bactericides.* LOUIS GERSHENFELD AND BERNARD WITLIN, Philadelphia College of Pharmacy and Science, Department of Bacteriology, Philadelphia, Pa.

No single laboratory test exists for determining the efficacy of an antiseptic, applicable under all conditions. A combination of *in vitro* and *in vivo* data is more useful in evaluating the effectiveness of an antiseptic. For obtaining combined data, a consideration of the egg injection method as the *in vivo* test and the FDA technique as the *in vitro* procedure is presented. A number designated as the "toxicity index" was determined from a comparison of the bactericidal efficiency against *Staphylococcus aureus*, with the toxicity of the bactericide for chick embryos.

$$\text{Toxicity Index} = \frac{\begin{array}{l} \text{Actual amount of bactericidal agent in 1 ml} \\ \text{of the highest dilution, killing } S. \\ \text{aureus in 10, but not in 5, minutes at 37 C.} \end{array}}{\begin{array}{l} \text{Minimum amount of bactericidal agent killing 9-day-} \\ \text{old chick embryo within 24 hours.} \end{array}}$$

Toxicity indices were determined using seventeen commonly employed antiseptics.



tics, representatives of the halogenic, mercurial, silver salt, phenolic, and quaternary ammonium compounds. The most favorable findings among the aqueous solutions of antiseptics were obtained with iodine (aqueous 2 per cent). The toxicity index was 0.0125. Among the tinctures, iodine (2 per cent in diluted alcohol) revealed the lowest toxicity index, 0.18. The mercurial and phenolic compounds displayed high toxicity indices.

*A43. Some Cultural and Biochemical Characteristics of a Thermophilic Cellulose Decomposer.* RICHARD H. MCBEE, State College of Washington, Division of Industrial Research, Pullman, Wash.

Thermophilic cellulose-decomposing bacteria have been obtained in pure culture. They have maintained their ability to digest cellulose through many transfers in cellulose as well as in sugar media. One culture ferments cellulose and cellobiose but does not utilize glucose. The nutritional requirements and the fermentation products have been studied.

*M1. Apparatus for Estimating Size of Bacteria-laden Airborne Particles.* W. F. WELLS, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

The collection of airborne particles impinged upon an agar surface by a high-velocity jet has been carefully studied by Bourdillon, Lidwell, and Thomas, in designing their slit sampler. The smallest bacteria-laden particles are collected with jet velocity of 270 feet per second. Efficiency decreases rapidly with decreasing jet velocity. An air centrifuge attached to the sieve modification of the slit sampler collected from the air passing the sieve many times more droplet nuclei, atomized from a 1 per cent suspension of a broth culture by a Walton humidifier, than were collected by the sieve. The efficiency of the sieve relative to the centrifuge, however, was increased by increasing air flow one-half. Droplet nuclei produced by natural sneezing have been studied by the sieve-centrifuge combination. Alpha hemolytic streptococci normally present in a nasopharynx were collected by the sieve on gentian violet blood agar plates and by the centrifuge in Difco air enrichment broth. Aliquots of the broth were divided into sterile tubes and the streptococci estimated by the dilution method, as in water analysis. Bacteria-laden nuclei sneezed into the air are somewhat larger than *Escherichia coli* suspended by the water humidifier. For field work in sanitary ventilation, this instrument is now being built with a small fan blower. Apparatus and results are exhibited.

*M2. A Method for Counting Bacteria in the Nasal Cavity: Application of the Method in Demonstrating the Action of Intranasal Penicillin Medication.* THOMAS C. GRUBB AND BRUNO PUETZER, Vick Chemical Company, Research Laboratories, Flushing, N. Y.

In order to determine the antibacterial activity of intranasally applied antibacterial agents, it is necessary to employ a uniform method of sampling the nasal flora at repeated intervals. The usual method of studying the types of

bacteria in the nasal cavity by inserting and culturing a cotton swab is not satisfactory for a quantitative determination of the number of bacteria present. To obtain a more accurate enumeration of bacteria in the nasal cavity the following method was used: A simple sampling device consisting of a test tube with an opening in the bottom which is connected to a nasal "olive" by rubber tubing was used to irrigate the nasal cavity with Ringer's solution. The washings were diluted quantitatively and cultured in blood agar plates. Each sample consisted of three consecutive washings made at 5-minute intervals which were pooled. The reliability of the method was tested by determining the nasal bacterial counts of five healthy subjects before, during, and after the application of penicillin nose drops. No carrying over of penicillin into the culture plates was detected. Although considerable daily variations in the counts on the same subject were found, the method was satisfactory for demonstrating the effectiveness of intranasally applied antibacterial agents. This method permits a qualitative as well as a quantitative examination of the nasal flora. While penicillin nose drops produced a marked reduction in the nasal bacteria of healthy subjects, this is not to be construed as a direct or implied indication of the efficacy of penicillin nose drops in nasal, sinus, or other respiratory infections.

*M3. Phagocytosis of Virulent Encapsulated Bacteria in the Absence of Antibody.*

MARY RUTH SMITH AND W. BARRY WOOD, JR., Washington University  
School of Medicine and Oscar Johnson Institute for Medical Research,  
Department of Medicine, St. Louis, Mo.

Results of a recent study of experimental pneumonia reveal, contrary to the accepted immunological concept, that phagocytosis of virulent encapsulated bacteria can occur in the absence of antibody, with subsequent bactericidal effect. This nonantibody mechanism operates in pneumonic, normal, and formalin-fixed lungs, on surfaces of tissues and inert materials as filter paper, but not on smooth surfaces such as glass. No intermediary opsonin is involved, the phagocytosis depending upon the physical properties of the surface contacted by the leucocytes. Direct visualization reveals that the "surface phagocytosis" depends upon leucocytes' trapping the bacteria against the surface. Intercellular "surface phagocytosis" can be demonstrated by merely concentrating leucocyte-bacterial mixtures by centrifugation. Under these conditions direct visualization reveals that the encapsulated bacteria are trapped between the surfaces of adjacent cells and are thus phagocyted in absence of opsonin. "Surface phagocytosis" of pneumococci and Friedlander's bacilli brings about rapid killing of the organisms. Digestion of intracellular pneumococci can be observed directly, and phagocyted Friedlander's bacilli, even when liberated from the cytoplasm of the leucocytes within 30 minutes, are nonviable. The nonantibody phagocytic mechanism appears to play a significant part in the recovery mechanism in both pneumococcal and Friedlander's pneumonia. Preliminary experiments indicate that "surface phagocytosis" operates not only in the lungs but also in other organs, thus constituting an important natural defense of body tissues in general against invasion by encapsulated bacteria.

*M4. The Relationship Between Ascorbic Acid and Phagocytic Activity.* ADA MAY AMES AND W. J. NUNGESTER, University of Michigan, Department of Bacteriology, Ann Arbor, Mich.

Results of a study of the relationship between ascorbic acid content of exudative polymorphonuclear leucocytes from guinea pigs and the fragility and phagocytic activity of these cells are presented. A parallel is also drawn between the level of vitamin C in the exudative cells and the quantity and quality of exudate obtained following injection of an irritating substance into the peritoneal cavities of guinea pigs. During these studies the vitamin C content of the exudate varied between 0 and 1.25 mg per 100 ml of exudate. Within these limits, with serum present in the system, the percentage of cells showing phagocytosis bears a direct ratio to ascorbic acid content. With levels from 0 to 0.25 mg per 100 ml, only 30 to 35 per cent of the cells show phagocytosis; with 1 to 1.25 mg per 100 ml of exudate, 80 to 90 per cent of the cells are active. Serum in the system, not only promotes phagocytic activity, but also tends to protect the cells from rupture. The amount necessary to afford such protection to the cells bears an inverse relationship to the vitamin C content of the cells. When the ascorbic level equals 0 to 0.25 mg per 100 ml, at least 15 per cent serum is necessary; with 1 to 1.25 mg only 5 per cent is needed to prevent rupture of the cells. When serum is lacking from the system, the fragility of the cells bears an inverse relationship to the ascorbic acid level of the exudate. With 0 to 0.25 mg present 90 to 95 per cent of the leucocytes were ruptured, while a 1 to 1.25 mg level resulted in only a 5 to 10 per cent rupture of the phagocytic cells.

*M5. The Inhibitory Action of Saliva on the Diphtheria Bacillus: Hydrogen Peroxide, the Antibiotic Agent of Salivary Streptococci.* RICHARD THOMPSON AND ANN JOHNSON, University of Colorado School of Medicine, Department of Bacteriology, Denver 7, Colorado.

Since the inhibitory action of saliva on *Corynebacterium diphtheriae* was shown to be due to viridans streptococci, we studied the role of hydrogen peroxide in this inhibition and the role of catalase in the staphylococcal antagonism of it. Drops of materials to be tested were placed on four plates containing diphtheria bacilli. Hydrogen peroxide was detected by benzidine or *o*-tolidine and potato. Catalase was detected by breakdown of hydrogen peroxide and recognized by gas production or by titration by permanganate. Seventy-one strains of salivary streptococci produced peroxide and inhibited diphtheria bacilli. The degree of inhibition was correlated with the concentration of peroxide produced. Several strains grown in deep broth produced no peroxide and filtrates of these cultures were not inhibitory. The same cultures, shaken for 10 minutes while exposed to air, produced large amounts of peroxide and their filtrates inhibited the bacilli. Seventeen strains of streptococci produced neither detectable peroxide nor appreciable inhibition. Thirty-four strains of staphylococci produced neither inhibition nor peroxide but produced catalase and antagonized the inhibitory actions of saliva and streptococci. Potato juice and lysed erythrocytes also antagonized these actions. Additional strains of diphtheria bacilli, pathogenic and nonpathogenic staphylococci, and enteric bacilli were tested

for inhibition by saliva, streptococci, and peroxide. The order of sensitivity was the same to all three agents, diphtheria bacilli being the most sensitive and enteric bacilli the least. The inhibitory action of saliva against diphtheria bacilli is largely due to hydrogen peroxide produced by the salivary streptococci.

*M6. Pork as a Factor in the Antigenicity of Avirulent Diphtheria Bacilli.* ELAINE L. UPDYKE AND MARTIN FROBISHER, JR., Johns Hopkins University, Department of Bacteriology, School of Hygiene and Public Health, Baltimore, Md.

In several experiments at different times 94 rabbits were repeatedly inoculated with living cultures of avirulent *Corynebacterium diphtheriae*. Twenty-two animals received organisms which had been cultivated in pork infusion broth. Of these 22, 8 (36 per cent) survived a subsequent challenge dose of virulent *C. diphtheriae* which was uniformly fatal to nonimmunized animals. The other 14 animals survived an average time of 5.3 days as contrasted to the 2.9 average survival time of 31 control (nonimmunized) animals. Seventy-two of the 94 animals received inoculations of avirulent *C. diphtheriae* cultivated on media not made with fresh pork, especially veal infusion broth. Of these 72 animals, none survived the challenge dose of virulent *C. diphtheriae*, and their average survival time (3.2 days) was essentially the same as that (2.9 days) of the 31 control animals. These results suggested that pork contains some factor which is critical for the protective antigenicity of avirulent diphtheria bacilli in regard to virulent diphtheria bacilli. Further experiments demonstrated that this factor is apparently not thiamine. The average survival time of 20 rabbits receiving low thiamine antigen was 3.4 days; that of 13 rabbits receiving high thiamine antigen was 2.5 days.

*M7. Fractionation of Hemolytic Streptococci by High-Speed Centrifugation, and Complement-Fixation Tests Between Nucleoprotein Constituents and Sera of Rheumatic Patients.* T. N. HARRIS, University of Pennsylvania (The Children's Hospital of Philadelphia), Department of Pediatrics, Philadelphia, Pa.

As the beginning of an analysis of the relation of the hemolytic streptococcus to rheumatic fever, group A hemolytic streptococci have been disintegrated by sonic vibration and then separated into chemically and serologically distinct parts by centrifugation at appropriate speeds, without use of acid or alkali. Three fractions have been obtained: (1) the low-speed sediment containing the hulls of the organisms, with the type-specific M-protein present; (2) cytoplasmic particles, largely sedimented at 15,000 rpm, which contain species-specific nucleoprotein and the group-specific carbohydrate; (3) a nucleoprotein, distinct from that of the cytoplasmic particles, not sedimented by speeds of 32,000 rpm. Each of the two somatic nucleoproteins have been separated into protein and nucleic acids, and the latter, which are serologically inert, have been identified. Both of the nucleoproteins are antigenic, and complement-fixation tests have been carried out with each against sera of immunized rabbits,

normal human beings, patients with acute streptococcal disease, and patients with rheumatic fever in various stages of activity. Parallel determinations of the antistreptolysin titers have been made with these sera.

*M8. Experimental Streptococcal Infections. II. A Study of Spreading Factors Produced by Hemolytic Streptococci.* NOBLE P. SHERWOOD AND BARBARA E. RUSSELL, University of Kansas, Department of Bacteriology, Lawrence, Kans.

In a previous communication one of us reported upon the ability of representative strains of the various Lancefield groups of hemolytic streptococci to spread within the mesoderm of the C-A membrane of the embryonic chick. The present paper is a report of our studies of the filtrates of these and other hemolytic streptococci, representing nine Lancefield groups, to determine whether they could reduce the viscosity of hyaluronic acid and could spread within the dermis of albino rabbits. The effect on the streptococci of animal passage was investigated. The results may be summarized as follows: Filtrates possessing the property of reducing the viscosity of hyaluronic acid solutions were found only among cultures of some members of Lancefield groups A, B, and C, the majority of group A strains giving negative results. All filtrates that reduced the viscosity of hyaluronic acid spread rapidly when injected into the skin of rabbits. A number of filtrates of organisms belonging to groups A, B, C, and D did not reduce viscosity of hyaluronic acid solutions but did spread slowly in the skin of rabbits. When strains were passed through mice and rats, it was observed that occasionally there was an apparent loss of ability to reduce the viscosity of hyaluronic acid solution although there was frequently an apparent acquired ability to spread within the skin. Cyanide, iodine, fluoride, peroxide, and shaking did not inactivate hyaluronidase. Heating to 50 C for 1 hour completely inactivated the bacterial enzyme and reduced the activity of the testicular enzyme.

*M9. The Collagenase Activity of Culture Filtrates of Clostridium histolyticum.* MARSHALL W. JENNISON, Syracuse University, Department of Plant Sciences, Division of Bacteriology, Syracuse, N. Y.

In a previous paper on the action of several bacterial species on collagen, it was noted that bacteria-free filtrates from cultures of *Clostridium histolyticum* showed a high collagenase activity. The present report deals with this activity quantitatively, with regard to age of culture and rate of digestion of the collagen. Culture filtrates of different ages were prepared by growing the organism in liquid thioglycolate medium (without glucose or agar) at 37 C for 24, 48, 72, and 96 hours, and filtering through a Seitz filter. The collagen substrate consisted of fine strands of clean, unprocessed beef tendon (about 85 per cent collagen), both unsterilized and sterilized (dry heat). Ten ml of culture filtrate (pH 7.2) and about 300 mg of tendon were used for each activity determination. The mixtures were incubated with and without toluol at 37 C for 24, 48, 72, and 96 hours, then filtered through quantitative filter paper, and the undissolved

collagen was weighed after drying. Enzyme activity was measured by digestion of the collagen, expressed as percentage loss in weight. The results show that filtrates from 24-hour cultures have a greater collagenase activity than those from older cultures. With 24-hour culture filtrates, a typical series gave 60, 83, 90, and 90 per cent of the collagen digested after 24, 48, 72, and 96 hours, respectively.

*M10. Streptococcal Content of Lungs of White Mice Infected with Streptococcus hemolyticus After Influenza Virus.* HAROLD N. CARLISLE AND N. PAUL HUDSON, The Ohio State University, Department of Bacteriology, Columbus 10, Ohio.

The observation that influenza virus infection increased the susceptibility of white mice to *Streptococcus hemolyticus* (group C) was confirmed. Mice that had been inoculated with killed or viable influenza virus were inoculated subsequently with hemolytic streptococci and sacrificed 24 hours later. The bacterial content of their lungs was determined by plate count. In one set of experiments, the interval between viral and streptococcal inoculations was 2 days, the virus dosages were 0.2, 1.0, and 100 MLD, and the coccal dosage was 100 MLD. Both inocula were given by intranasal instillation under ether anesthesia. The streptococcal count was 10 times greater in those mice that previously received active virus than in those inoculated with killed virus, without respect to dosage of virus. In another set of experiments, the interval between inoculations was varied from 0 to 24 days, the virus dose was 0.2 MLD, and the streptococcal inoculum was 100 MLD. Tenfold differences in the bacterial count were found in those groups of mice inoculated at intervals of 4, 8, and 12 days between agents; and fewer or no differences were found in those inoculated at other intervals. These data provide additional information relative to the period of increased susceptibility to the streptococcus arising as a result of sublethal influenza virus infection.

*M11. An Experimental Investigation of the Pathogenicity of Diphtheroids Isolated from the Human Conjunctiva.* CHARLES WEISS AND MARION C. SHEVKY, Laboratory for Ophthalmic Research, Mount Zion Hospital, San Francisco, Calif.

Diphtheroids resembling *Corynebacterium xerose* that were isolated from the human conjunctiva grew well in a menstruum of mucin. Similarly, when suspended in this medium and inoculated into the anterior chamber of the eyes of albino rabbits, cultures retained their viability for several days, while in salt solution they were rapidly destroyed. Intraocular injections of albino rabbits with diphtheroids suspended in saline produced moderate inflammation of the uveal tract. When suspended in mucin, the reaction lasted longer, was much more severe, and was associated with an acute keratitis. The lesions usually regressed spontaneously within 2 or 3 weeks. While killed cultures of diphtheroids in saline produced mild inflammatory changes in the ciliary process which were seen in histologic sections, none were visible grossly. In a menstruum of mucin the inflammatory reaction was more severe. Since mucin by itself was relatively

innocuous, it is suggested on the basis of these studies and those of others that it protects bacteria from the digestive action of endocellular proteolytic enzymes and other immunologic defense mechanisms. Living diphtheroids are thus permitted to grow and exert their pathogenic activity. By applying the recently developed principles of Mueller and Miller and Pappenheimer and Johnson, it was possible to demonstrate that a filtrate which is produced by growing diphtheroids in a medium of very low iron concentration is injurious to the uvea and cornea, but not to the skin or conjunctiva, of albino rabbits. On the basis of these investigations, it may be concluded that diphtheroids which are present on the normal or the inflamed human conjunctiva may be considered as potential pathogens which may exert injury when they are introduced into the interior of the eye.

*M12. The Production of Salmonella Osteomyelitis in White Rats.* J. EMERSON KEMPF AND THEODORE A. FOX, University of Illinois College of Medicine, Department of Bacteriology and Public Health and Department of Orthopedic Surgery, Chicago, Ill.

Osteomyelitis in man caused by *Salmonella typhimurium* has been reported with increasing frequency. Therefore, it seemed advisable to produce salmonella osteomyelitis in a laboratory animal such as the white rat in order that more extensive studies of the disease might be made. The culture of *Salmonella typhimurium* used in these experiments was isolated from a bone lesion of a patient with chronic osteomyelitis and identified by serological methods. An incision was made over the proximal portion of the tibia of a white rat and a 2-mm opening was made into the bone marrow. A small pledget of cotton which had been immersed in a 24-hour broth culture of *S. typhimurium* was inserted into the bone and the incision was closed. Control animals received an insertion of cotton and sterile broth into the tibial bone marrow. The animals were observed for 21 days, at the end of which period the animals were sacrificed, and the lesions, the liver, and spleen were cultured; gross and microscopic examinations of the tibia were also made. Of 15 rats inoculated, 14 developed typical microscopical lesions of osteomyelitis and positive cultures of *S. typhimurium*. The spleens of three animals yielded the same organism. Twelve rats used as controls had no bone lesions, 11 had negative cultures, and 1 had a gram-positive coccus. It is concluded that osteomyelitis caused by *S. typhimurium* can be readily produced in white rats and that the rat is a suitable animal for the study of this disease.

*M13. Infection and Intoxication of Macacus mulatta with Shigella dysenteriae (Shiga).* SARA E. BRANHAM AND KARL HABEL, National Institute of Health, Public Health Service, Bethesda, Md.

Monkeys were fed cultures of mouse-virulent strains of *Shigella dysenteriae* (Shiga) in doses of 2 to 100 billion bacteria. Shiga organisms were recovered from stools on the following day only. No monkeys seemed ill. Other monkeys were injected intraperitoneally with Shiga cultures in mucin in doses of 10

million to 20 billion bacteria. Those receiving 10 million were unaffected. Those receiving 100 million showed malaise for 2 days but recovered. Those receiving 20 billion developed an acute hemorrhagic peritonitis and died within 24 to 72 hours. *S. dysenteriae* was recovered from heart blood and various organs with these fatal doses, but not from the blood of any that survived. Other monkeys were fed undiluted Shiga toxin. An increased excitability, leg muscle weakness, and, in one case, tetanic convulsions were the only effects produced. Monkeys injected with relatively high dilutions of toxin intravenously showed a characteristic picture. An incubation period of at least 48 hours was followed by weakness of the legs and by tetanic convulsions if the animal was exercised. In fatal intoxications the state of convulsions was followed by an ascending spastic paralysis, prostration, and death. Death occurred in from 48 hours to 4 to 5 days as a rule. At autopsy these animals showed hemorrhagic petechiae on the skin and hemorrhage into the lymph nodes, peritoneum, and walls of large intestine. Most conspicuous was hemorrhage into the heart muscle. Histological sections showed focal hemorrhages into many tissues, especially into heart muscle and adrenals. Sections from brain and cord were essentially negative. Apparently Shiga toxin is relatively innocuous to the unbroken intestinal mucosa but a small amount parenterally can produce a characteristic and rapidly fatal syndrome.

*M14. The Potential Pathogenicity of Bacillus cereus and Its Relationship to Bacillus anthracis.* KENNETH L. BURDON, Baylor University College of Medicine, Department of Bacteriology and Immunology, Houston, Texas.

The close similarity between *Bacillus anthracis* and *Bacillus cereus* extends to the possession of a definite pathogenicity for laboratory animals on the part of the latter organism. There is evidence also that *B. cereus* may cause anthrax-like lesions in man. Nevertheless, distinctive properties of *B. cereus* serve to differentiate it from both virulent and nonvirulent strains of the anthrax bacillus. Moderate doses of active, young cultures of *B. cereus* (which are markedly hemolytic) may cause local hemorrhagic and granulomatous lesions, or death within a few hours, in rabbits and guinea pigs. When broth cultures of such active strains are inoculated intraperitoneally into mice, in very small doses, corresponding to the minimal dose of freshly isolated, virulent anthrax bacilli required to cause death in 18 to 24 hours, they may produce an overwhelming septicemia, so that the mice die within 6 hours. Mice injected subcutaneously usually develop a large ulcer at the site of inoculation. These pathologic effects of *B. cereus* are enhanced by the hemolytic activity of the cultures, but they are due primarily to a true infective process; virulence is increased by animal passage. However, this pathogenic action still differs from the characteristic course of anthrax infection, and at autopsy the organisms, though numerous in the tissues, fail to show capsules. Attenuated strains of *B. anthracis* may not be identifiable by animal inoculation, but they are still distinguishable from *B. cereus* by differences in growth temperature limits, sensitivity to penicillin, hemolytic power, and several other easily demonstrated properties.



*M15. Estimation of LD<sub>50</sub> Titers of Psittacosis Lymphogranuloma Viruses in Embryonated Eggs.* ORVILLE J. GOLUB, Camp Detrick, Frederick, Md.

A true linear relationship has been shown to exist between the concentration of virus inoculated into the yolk sac of embryonated eggs and the resulting average day of death (A.D.D.) of the embryos. From composite data it has been possible to establish a plotted line from which an estimate of the LD<sub>50</sub> value of a virus suspension can be made by determining the A.D.D. of embryos inoculated with a single dilution and candled at regular 24-hour intervals. Lines have thus been plotted for two psittacosis viruses (6BC and Gleason strains), meningopneumonitis virus (Cal 10 strain), and Louisiana pneumonitis virus (Borg strain). The error of LD<sub>50</sub> estimates by this method is shown to be approximately the same as that which obtains in the standard titration method employing 10 to 20 eggs per dilution. Either 8- or 9-day-old embryos may be used with no significant change in titer resulting. The advantages of this method are: (1) fewer eggs and less time are required; (2) titrations are usually completed within 4 to 6 days instead of the usual 10; and (3) end points are not missed through improper choice of dilutions.

*M16. Studies on Lymphogranuloma Venereum Complement-fixing Antigens IV. Fractionation with Organic Solvents of Antigens of the Psittacosis Lymphogranuloma Venereum Group.* MAURICE R. HILLEMANN AND CLARA NIGG, E. R. Squibb and Sons, Virus Laboratories, New Brunswick, N. J.

Earlier studies had shown that a complement-fixing yolk sac antigen for lymphogranuloma venereum was soluble in ether. This study is concerned with the purification and the properties of the various fractions obtained by ether extraction of yolk sac antigens for the psittacosis lymphogranuloma venereum group of viruses. A high degree of purification was effected by successive extraction of the ether extract with acetone and methyl alcohol. The acetone- and alcohol-soluble fractions were inactive. The acetone-insoluble, alcohol-insoluble fraction, inactive as such, was fully activated by the addition of optimal amounts of lecithin of either yolk sac or vegetable origin. The ether-soluble antigen in the form of a saline suspension showed no loss in reactivity when stored at refrigerator temperature for more than 18 months. Chloroform extracts of infected yolk sac suspensions were similar to the ether extracts in that most of the activity was in the extract. Benzene and petroleum ether extracts of yolk sac suspensions showed little or no activity, whereas the suspensions after extraction showed markedly enhanced activity. No specificity within the psittacosis lymphogranuloma venereum group could be demonstrated with any of the fractions studied.

*M17. The Transmission of the Virus of Lymphocytic Choriomeningitis by Trichinella spiralis.* J. T. SYVERTON, O. R. MCCOY, AND J. KOOMEN, JR., University of Rochester School of Medicine and Dentistry, Department of Bacteriology, Rochester 7, N. Y.

The purpose of this laboratory investigation was to open further avenues for

the epidemiological investigation of natural virus transmission and of virus survival during interepidemic periods. The experiments were designed to learn whether the nematodal parasite, *Trichinella spiralis*, could act as an intermediary for the maintenance and transfer of the virus of lymphocytic choriomeningitis from one host to another. *T. spiralis* and lymphocytic choriomeningitis were selected because each is representative of a large class of closely related agents, each has a wide host range that includes man, and each is cosmopolitan in distribution. Conclusive results were obtained in three out of six experiments in which guinea pigs were infected concurrently with the virus of lymphocytic choriomeningitis and *T. spiralis*. They showed that trichinella larvae, after maturation in the muscles, had acquired the virus and were capable of transmitting it to new hosts. Transmission resulted when living larvae were fed to normal guinea pigs and when triturated dead larvae were injected. Virus harbored within the body of the larvae rather than virus on their outer surfaces was responsible for transmitting the infection. The results indicate that *T. spiralis* may act under experimental conditions as an efficient vehicle for the maintenance of the virus of lymphocytic choriomeningitis, for its penetration through natural barriers, and for its transmission to new hosts. The relationship of these results to the natural spread of lymphocytic choriomeningitis is unknown.

*M18. Isolation of the Virus of Herpes Simplex from Six Cases of Kaposi's Varicelliform Eruption.* ISAAC RUCHMAN, KATHARINE DODD, AND ASHTON WELSH, The Children's Hospital Research Foundation and The University of Cincinnati College of Medicine, Departments of Bacteriology, Pediatrics and Dermatology and Syphilology, Cincinnati, Ohio.

Herpes simplex virus was recovered by inoculation of the rabbit's cornea with material obtained from the skin lesions of three children and three adults presenting the syndrome of Kaposi's varicelliform eruption. The presence of intranuclear inclusion bodies of the herpetic type in the lesions of the rabbit cornea and the demonstration of cross immunity in animals with a known strain of herpes virus (H. F. strain) proved the identity of the agent recovered. An increase in antibodies against the homologous strain of virus was demonstrated in the sera of all three children and of one adult patient during convalescence. Antibodies were absent in the serum of one fatal adult case. In the serum of the third adult antibodies were found during the acute phase of the illness; no rise in antibody titer subsequently occurred. All six patients had been in contact with individuals suffering from labial herpes 5 to 10 days preceding the onset of this varicelliform eruption but gave no history of exposure to vaccine virus.

*M19. A Quantitative Method for the Assay of Influenza Virus Vaccine, Influenza Serum, and Complement.* JAMES W. FISHER, Department of National Health and Welfare, Laboratory of Hygiene, Ottawa, Canada.

Studies were made to develop a method that may be employed for the quanti-

tative evaluation of influenza virus vaccine, influenza serum, and complement. The activity of one or more specimens of these materials may be assayed in terms of standard preparations by the use of an *in vitro* assay method. When influenza virus vaccines or sera are titrated by the method of Hirst and Pickels, a straight line is obtained by plotting either probit or logit percentage maximum sedimentation of the cells against the logarithm of the dilution of the test reagents. By this procedure one may determine the relative value of two preparations and estimate the degree of precision of the results. For example, a serum having a theoretical strength of one-third of the reference standard was found by actual test to have a potency of  $35.0 \pm 4.1$  per cent of the latter at  $P = 0.05$ . Similarly, in the assay of complement in the complement-fixation test, a linear dosage response relationship may be used by plotting either probit or logit percentage hemolysis against the logarithm of the amount of complement. By experiment a preparation of complement was found to be  $50.7 \pm 1.4$  per cent ( $P = 0.05$ ) of the standard rather than the theoretical 50 per cent. The hemolysin was assayed by the slope ratio method. In tests using positive and negative typhus or encephalitis antigens and sera, parallel lines were obtained that differed significantly in position. Smaller amounts of antibody were assayed by this method than by other complement-fixation techniques.

*M20. Studies on the Propagation of PR8 Influenza Virus in the Developing Chick Embryo.* E. B. MCQUARRIE, D. PERLSTEIN, AND A. J. LIEBMANN, Schenley Distillers Corporation, Larchmont Research Laboratories, Larchmont, N. Y.

With the desire to use the techniques of chick embryo propagation and the Hirst and Pickels' method of determination of influenza virus for studies on virus inhibitors, a study of these methods has been made. To increase the accuracy of the virus determination and to speed the calculation of results, it has been found desirable to use a daily virus hemagglutination standard curve. With a standardized procedure for the production of influenza virus in the chick embryo, the standard deviation of titer values ranged from 25.5 to 69 per cent of the average titer for replicate embryos, and was 16 per cent of the average titer obtained from pooled lots of allantoic fluid from groups of 10 to 20 embryos. The mechanism of virus multiplication in the embryo is remarkably unaffected by the addition of foreign substances. The addition of relatively high concentrations of malonate and thiourea into the allantoic cavity of the embryo caused a reduction in virus multiplication, while cyanide, fluoride, and oxalate were ineffective. The large inherent variation in replicate embryos is demonstrated by the large variations in pH, volume, and uric acid content of the allantoic fluid. The variation in pH is partially explained by the low buffer power of the fluid between pH 7.5 to 8.5. The retarding of embryo development by virus multiplication is indicated by the lower uric acid concentration in the allantoic fluid of infected embryos. The relationship between pH and virus concentration of infected allantoic fluid will be discussed.

*M21. Studies on Interaction of Red Cells and Influenza Virus with Electron Microscope.* F. HEINMETS, Camp Detrick, Frederick, Md.

Active and inactive influenza virus was used for interaction studies with chick red cells and human red cell ghosts. Observations with the electron microscope were made during the various stages of interaction, demonstrating absorption of virus particles on red cell surface and agglutination phenomena. Studies were made also on aggregation and disintegration of virus particles as a function of time. It was observed that virus particles in filament form (possibly due to polymerization) were absorbed on the red cell surface.

*M22. Preliminary Studies on the Identification of the Principle in Chorioallantoic Fluid Responsible for the Agglutination of Some Strains of Staphylococcus aureus.* EDWARD W. SHRIGLEY AND ESTHER S. MACULLA, Yale University School of Medicine, Departments of Bacteriology and of Pathology, New Haven 11, Conn.

Chorioallantoic fluid from normal hens' eggs as well as that from eggs infected with influenza virus A (PR8) will agglutinate certain strains of *Staphylococcus aureus*. The present study is an effort to determine the nature of the agglutinating principle in these fluids. It has been demonstrated that virus-infected fluids possess a greater capacity to elicit the phenomenon than normal (non-virus-infected) materials. However adsorption and elution studies suggest that the virus per se has nothing to do with this reaction. The agglutinating principle is thermolabile, being destroyed at 56 C in 2 to 5 minutes. Its activity is favored by an alkaline reaction, but acidification does not destroy the principle, since an increase in pH will restore activity. The substance is not dialyzable either through cellophane or collodion membranes. It is insoluble in ether and acetone, and appears to be inactivated by alcohol. Evidence suggests that the agglutinating principle will precipitate out with ammonium sulfate saturation of the fluid; it appears to be present in the white sediment which forms either on long standing or following freezing and thawing. It is possible to concentrate the substance by lyophilization and ultrafiltration. The effect of proteolytic enzymes on the principle is now under study.

*M23. Nonspecific Inhibition of Virus Hemagglutination.* W. F. FRIEDEWALD, E. S. MILLER, AND L. R. WHATLEY, Emory University School of Medicine, Department of Bacteriology and Immunology, Atlanta, Ga.

The agglutination of red blood cells by certain viruses may be inhibited by a variety of materials not containing specific antibody, such as normal animal sera and allantoic fluid. In a study of this nonspecific inhibition reaction, sera and tissue extracts of human and animal origin were tested by means of a modified Salk method, using mumps virus and the PR8 and Lee strains of influenza virus. Inhibition of hemagglutination was obtained in high titer with saline extracts of organs (lungs, liver, kidney, spleen) from human autopsies and from normal rabbits. The serum inhibition titers were invariably less.

Saline extracts of human and chicken red blood cells caused inhibition in high titer, in contrast to low titers found in sheep and rabbit red cell extracts. When the virus receptor substance was removed from human and chicken red cells by Hirst's method of adsorption and elution, extracts of these cells no longer contained the inhibitory substance. Furthermore, some virus was released from its union with the inhibitory substance after incubation for 6 hours at 22 C or 37 C. The substance did not neutralize influenza virus in mice and it failed to fix complement in mixture with influenza or mumps virus. The findings indicate that the inhibitory factor is the virus receptor substance which has been released from cells.

*M24. A Method for Evaluation of Influenza-Virus-inhibiting Substances.* A. J. LIEBMANN, D. PERLSTEIN, AND G. A. SNYDER, Schenley Distillers Corporation, Larchmont Research Laboratories, Larchmont, N. Y.

The Hirst method for determination of influenza virus has been primarily applied to the quantitative assay of the virus and the estimation of antibody titers. In the search for substances possessing qualities of inhibiting influenza virus, the Hirst method has been adapted and amended for the evaluation of such virus-inhibiting substances in the chick embryo. The tests were conducted with a substance so far not isolated or identified, and in concentrations which are unknown and probably were not constant in the various determinations. Inhibition both *in vitro* and *in vivo* has been quantitatively determined in a significant number of instances. A quantitative relation has been established and measured by this method between the inhibitive qualities of an antiviral substance and influenza virus at various degrees of concentration. The unknown substance is obtained as the metabolic fermentation product of a microorganism isolated from soil, but not as yet identified. Results are reported in tables and graphs from more than 250 fermentations.

*M25. Inhibition of Hemagglutination and of Multiplication of Influenza Virus by Certain Polysaccharides.* D. W. WOOLLEY AND R. H. GREEN, Rockefeller Institute for Medical Research, Department of Physiology, New York 21, N. Y.

If one assumes, as Hirst has done, that in causing hemagglutination influenza virus behaves as an enzyme which attacks a special substrate in the red cell, then it should be possible to inhibit the action of the virus on the erythrocyte by adding a suitable structural analog of this substrate. Since present evidence indicated that this substrate in the cell was carbohydrate in nature, a number of carbohydrates were tested for ability to prevent hemagglutination by influenza A virus. When judged by the appearance of the sedimented erythrocytes, several polysaccharides such as apple and citrus pectins, flax seed mucilage, myrrh, and gum acacia prevented the formation by the virus of characteristic patterns of agglutinated erythrocytes. Many other complex or simple carbohydrates were unable to do this, e.g., starch, oxidized starches, galacturonic or cellobiuronic acids, etc. The most effective agents were polygalacturonides, although complexes containing glucuronic or mannuronic acids had some potency. The

action appeared to be due to an exclusion of the virus from the erythrocyte, presumably by competition. Since it was assumed that hemagglutination is analogous to the initial stage of infection, the study was extended to a more complex system, namely, the infection of embryonated eggs. Apple pectin was found to inhibit or prevent multiplication of influenza A virus in the allantoic sacs of 10-day eggs. Thus, embryos injected with pectin and virus showed little or no multiplication of virus, as measured by both hemagglutination and infectivity.

*M26. Effect of Acridines on the Growth of Influenza A and B Viruses.* A. F. RASMUSSEN, JR., JULIA C. STOKES, HARRY A. FELDMAN, AND JOSEPH E. SMADEL, Army Medical Department Research and Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

A preliminary report from this laboratory has been published on the inhibiting action of 2,3-dimethoxy-6-nitro-9-(diethyl-amino-oxypropyl)aminoacridinedihydrochloride, nitroakridin 3582, on the growth of type B (Lee) influenza virus in embryonated eggs. Further experiments in which parallel infectivity and red cell agglutination titrations were made on allantoic fluids taken from treated eggs at intervals after inoculation with Lee influenza virus confirmed and extended the earlier observations. The administration of 0.5 to 1.0 mg of nitroakridin 3582 prior to inoculation with 10 minimal infecting doses of virus resulted in varying degrees of suppression of virus growth. Virus was either absent or present in low concentrations in the allantoic fluids of most treated eggs throughout the period of observation, 48 to 72 hours. In certain eggs the appearance of virus was delayed 8 or more hours, but it ultimately reached a concentration equal to that in untreated controls. Nitroakridin 3582 has a similar but less pronounced effect on type A (PR8) influenza virus infections in embryonated eggs. A group of related compounds have also been investigated and rutenol, 2-nitro-5-aminoacridine, and atabrine have been found to have some viristatic activity when tested against the influenza viruses. Nitroakridin 3582 is somewhat viricidal *in vitro* but this is insufficient to account for the degree of inhibition observed *in vivo*.

*M27. Nonbacillary Forms of Mycobacterium tuberculosis and Mycobacterium leprae.* ELEANOR ALEXANDER-JACKSON, Cornell University Medical College, Department of Public Health and Preventive Medicine, New York 21, N. Y.

In 1945 the writer described a hitherto undemonstrated form of *Mycobacterium tuberculosis* as revealed in both unstained cultures and material stained by a new differential triple stain technique. A study of skin biopsies from leprosy lesions stained by this method revealed not only non-acid-fast rods and granules but non-acid-fast and semi-acid-fast zooglyphic forms identical to those observed for *Mycobacterium tuberculosis*. Recently, a comparison of slides from treated and untreated leprosy lesions disclosed that in treated lesions, both rod and

zooglear forms—if not fragmented or destroyed—become condensed by degrees into large numbers of non-acid-fast or semi-acid-fast globoid bodies. Similar globoid condensation bodies were also observed in both stained and unstained preparations from cultures of H37RV tubercle bacilli in Dubos medium containing various concentrations of streptomycin, in fluid from streptomycin-treated tuberculous patients, and in preparations from tissues and fluids of immunized tuberculous guinea pigs. The interrelationship of the various forms of these mycobacteria is being studied.

*M28. The Effect of Tissue Extracts on Experimental Tuberculous Infections in Mice.* GIRARD W. THOMAS AND LEO G. NUTINI, Institutum Divi Thomae, Department of Experimental Medicine, Cincinnati 6, Ohio.

Studies using deproteinized aqueous extracts of beef brain, heart, liver, kidney, and spleen, previously shown to have bacteriostatic action on the growth of the tubercle bacillus in Youmans' liquid synthetic medium, were extended to the H37Rv strain. Heart extract, which in 5 per cent concentration had shown bactericidal activity for the H37 strain, was likewise bactericidal for the H37Rv type within 21 days. For *in vivo* experiments, 20 mice were used as control animals, and 20 for each extract, with the exception of brain extract, for which 10 mice were used. One-tenth mg of the H37Rv strain was injected intravenously. After 28 days' treatment with spleen and heart extract (20 mg per day subcutaneously) the mortality rate was 15 and 10 per cent respectively, and 60 per cent in the control series. Mortality with the brain, kidney, and liver extracts was 40, 35, and 45 per cent respectively. In untreated control mice, the lungs showed massive tubercle formation and marked enlargement. In those treated with heart and spleen extracts, there was slight enlargement with little evidence of tubercle formation. Acid-fast bacilli were found in the lungs of the control animals in large numbers. They were slightly reduced in those treated with spleen extract, whereas the lungs of the mice receiving the heart extract contained only a few isolated organisms. Kidney, brain, and liver extracts appeared to have no influence on the number of organisms present in the lung tissue.

*M29. The Effect of Para-aminosalicylic Acid on Tubercle Bacilli.* GUY P. YOUNG AND GORDON W. RALEIGH, Northwestern University Medical School, Department of Bacteriology, Chicago, Ill.

Para-aminosalicylic acid was found to be highly bacteriostatic *in vitro* for 17 strains of virulent human type tubercle bacilli, including six strains resistant to the bacteriostatic action of streptomycin. This tuberculostatic activity was partially reversed by *p*-aminobenzoic acid, but was not markedly interfered with by the presence of plasma. When *p*-aminosalicylic acid, either in the form of the hydrochloride or the free base, was administered by the drug diet method to white mice infected intravenously with 0.1 mg of the virulent H37Rv strain of *Mycobacterium tuberculosis*, it was found to exert a suppressive action on the

tuberculous process. Toxic reactions were noted in the mice when the compound was administered in the diet in concentrations of 2.0 and 4.0 per cent, but were not evident when administered in a concentration of 1.0 per cent.

*MS0. Comparative Susceptibilities of Different Strains of Mice to Experimental Infection with Mammalian Tubercle Bacilli.* CYNTHIA PIERCE, RENE J. DUBOS, AND GARDNER MIDDLEBROOK, Rockefeller Institute of Medical Research, Department of Pathology and Bacteriology, New York, N. Y.

Mice of known genetic backgrounds were infected with young cultures of tubercle bacilli growing diffusely in the tween-albumin liquid medium. Striking and reproducible differences were observed in the course and outcome of the infection, resulting from injection of small amounts of bacilli by the intraperitoneal, intravenous, or intracranial routes. Of the 20 strains tested, mice derived from the Albino Swiss exhibited highest resistance, whereas all pigmented strains were more susceptible.

*MS1. The Effect of Diet on the Susceptibility of Mice to Experimental Infection with Mammalian Tubercle Bacilli.* RENE J. DUBOS AND CYNTHIA PIERCE, Rockefeller Institute for Medical Research, Department of Pathology and Bacteriology, New York, N. Y.

The susceptibility of mice to experimental tuberculous infection varies greatly, depending upon the composition of the diet. Highest susceptibility was observed when the animals were maintained for 2 weeks on a mixture of corn meal, gelatin, butter fat, nicotinic acid, and minerals. Resistance was much increased when certain natural food products were added to this mixture.

*MS2. Differential Characteristics of Virulent and Avirulent Variants of Mammalian Tubercle Bacilli.* GARDNER MIDDLEBROOK, RENE J. DUBOS, AND CYNTHIA PIERCE, Rockefeller Institute for Medical Research, Department of Pathology and Bacteriology, New York, N. Y.

Virulent mammalian tubercle bacilli multiplying in infected tissues or in tween-albumin liquid medium grow in the form of long strands consisting of strongly acid-fast cells oriented in parallel along their longitudinal axis. The avirulent bacilli, on the contrary, are less uniformly acid-fast and occur in clumps without any polarity; the individual cells in these clumps exhibit random orientation or, at most, a suggestion of rosette formation. These differences in microscopic cellular arrangement are reflected in the gross macroscopic appearance of the growth of the cultures. On the surface of liquid media, the virulent culture spreads to form a thin veil which rapidly and uniformly covers the whole surface, whereas the avirulent culture gives rise to isolated islands of growth, presenting a granular and clumpy appearance. On tween-albumin agar, the colonies of the virulent form are flat, translucent, and spreading; the avirulent form, on the other hand, gives raised, opaque colonies with little tendency to spread peripherally.



*M33. Resistance of Tubercle Bacilli to Streptomycin in Guinea Pigs After Administration of the Drug: The Effect on Response to Treatment with Streptomycin.* ALFRED G. KARLSON AND WILLIAM H. FELDMAN, Mayo Foundation, Division of Experimental Medicine, Rochester, Minn.

Twenty-eight guinea pigs were each inoculated with 0.1 mg of tubercle bacilli resistant to only 0.15 micrograms of streptomycin per milliliter of medium. Twenty days later four were killed and found to have visible lesions of tuberculosis. At this time treatment of ten of the remaining guinea pigs with streptomycin was started. Each received 1.5 mg four times daily. The other 14 animals served as controls. Treatment had continued for 146 days when the experiment was ended on the one hundred and sixty-sixth day of infection. The untreated guinea pigs survived an average of only  $70.6 \pm 22$  (standard deviation of the mean) days. All had widespread lesions of tuberculosis. When cultures from their spleens were made, the same sensitivity to streptomycin was noted as in the original culture. Two of the treated animals died on the one hundred and sixtieth and one hundred and sixty-fourth day, respectively, from causes other than tuberculosis. The other eight were apparently well on the one hundred and sixty-sixth day after having been treated for 146 days. They were then killed and three were found to have visible lesions. Organisms in cultures from the spleens of these were resistant to more than 2,000 micrograms of streptomycin per milliliter of medium. Seven of the treated animals had no lesions of tuberculosis. On culture of the spleens from two, no growth was observed in 60 days. Organisms in cultures from the other five were resistant to only 0.15 micrograms of streptomycin. Histologically little evidence of tuberculosis was found in any organ; in the three treated animals from which resistant strains of tubercle bacilli were isolated, the tuberculous process of recent origin was unrestricted.

*M34. Tuberculostatic and Tuberculocidal Action of Streptomycin.* DOROTHY G. SMITH AND SELMAN A. WAKSMAN, New Jersey Agricultural Experiment Station, Department of Microbiology, New Brunswick, N. J.

By the use of the turbidimetric procedure for measuring diffuse growth of *Mycobacterium tuberculosis* in Dubos' medium, it is possible to study accurately the bacteriostatic activity of various antibiotic substances, especially streptomycin. The tuberculocidal action of this antibiotic was determined by plating procedures, using the above medium with agar. Streptomycin, in a concentration of 0.3 micrograms per ml, was completely bacteriostatic, although occasionally growth occurred even in concentrations of  $0.5 \mu\text{g}$  per ml. The bactericidal effect was greatly influenced by the incubation period. Only  $0.3 \mu\text{g}$  per ml of streptomycin was required for the killing of all cells of a given inoculum if incubation was continued for 48 hours, whereas  $20 \mu\text{g}$  per ml were needed when the culture was incubated only 6 hours. The degree of activity of streptomycin upon the tubercle bacilli was found to be a function of the number of cells and the length of time of exposure of the cells to the antibiotic.

*M35. Some Biological Properties of Proteins from Unheated Tubercle Bacillus Culture Filtrates.* JANET R. McCARTER AND ELLEN B. BEVILACQUA, The University of Wisconsin, Department of Agricultural Bacteriology and Chemistry, Madison, Wis.

By means of quantitative precipitin and precipitin absorption tests and of physicochemical methods, a survey has been made of all fractions precipitable by  $(\text{NH}_4)_2\text{SO}_4$  from *unheated* culture filtrates of the human tubercle bacillus. Two protein fractions were found to be distinct and homogeneous, both according to their sedimentation constants and to their behavior as precipitinogens. The one antigen, previously found by Seibert, came from a fraction precipitated by one-fourth saturation with  $(\text{NH}_4)_2\text{SO}_4$  and had a sedimentation constant of about 3.5S. The other was precipitable by saturation with  $(\text{NH}_4)_2\text{SO}_4$  and had a constant of about 2.0S. These two antigens were the only ones detected in culture filtrates of one strain of avirulent tubercle bacillus. A third antigen was found associated with the other two in certain fractions from a virulent strain. This "virulence" antigen was by far the best precipitinogen of the three antigens. All were good precipitants in comparison with other described bacterial proteins. Both the "one-fourth" and the "saturated" antigens were potent in eliciting skin reactions in tuberculous human beings and guinea pigs. The potency tests were made on individual animals since the simultaneous injection of the two proteins into guinea pigs sensitized with tubercle bacillus cells resulted in partial inhibition. Either of these two antigens injected intracutaneously into the normal guinea pigs caused sensitization so that subsequent injections gave skin reactions. Skin sensitivity to the homologous protein could be transferred passively to normal guinea pigs by intracutaneous injections of the serum from the sensitized animals.

*M36. Metabolic Changes in Certain Mycobacteria Associated with Development of Resistance to Streptomycin.* R. J. FITZGERALD AND F. BERNHEIM, Duke University School of Medicine, Department of Physiology and Pharmacology, Durham, N. C.

The growth of an avirulent strain of *Mycobacterium tuberculosis* (ATC 607) is completely inhibited by streptomycin in concentrations of 60 to 75 units per cent. Streptomycin also inhibits the growth of a saprophytic soil *Mycobacterium*, but concentrations of 125 to 150 units per cent are required. Both these strains can oxidize benzoic acid. Streptomycin in a concentration of 5.0 units per ml can completely inhibit the oxidation of benzoic acid by the 607 strain of *M. tuberculosis*, whereas 20 to 30 times that amount is necessary to inhibit this oxidation by the soil *Mycobacterium*. Streptomycin resistance was induced in these strains by growing them in increasing concentrations of the drug until they grew readily in the presence of 10,000 units per cent of streptomycin. With development of increasing resistance to streptomycin the sensitivity of the benzoic-acid-oxidizing mechanism to streptomycin decreases progressively, until finally, in strains resistant to 10,000 units per cent, the oxidation of benzoic acid is unaffected by large amounts of streptomycin.

*M37. A Quick Microtechnique for the Detection of Acetylmethylcarbinol Production by Bacteria.* ANGELINA FABRIZIO AND R. H. WEAVER, University of Kentucky, Department of Bacteriology, Lexington, Ky.

The principles of Weaver, Arnold, and Hannan (1946) have been used in the development of a quick microtechnique for the detection of acetylmethylcarbinol production by bacteria. An infusion medium containing 1 per cent trypticase, 0.7 per cent glucose, and 0.5 per cent sodium chloride is distributed in 0.5-ml amounts in 10 × 75 mm tubes and the tubes are preheated to 37 C. Each tube is inoculated with a loopful of growth from a 6- to 12-hour infusion agar slant culture. The tubes are placed in a water bath at 30 C for 90 minutes, after which time tests are performed for the detection of acetylmethylcarbinol by the addition of 0.15 ml of 5 per cent  $\alpha$ -naphthol solution followed by 0.05 ml of 40 per cent potassium hydroxide containing 0.3 per cent creatine. The tubes are shaken for 5 seconds after the addition of each reagent and returned to the water bath for 30 minutes. Of 408 recently isolated coliform cultures that produced acetylmethylcarbinol according to the Dorner and Hellinger procedure, 394 (96.5 per cent) yielded positive results by the quick microtechnique. Of the 14 that yielded negative results, 10 were atypical *Aerobacter aerogenes* according to their IMViC reactions. None of 372 cultures that were negative by the Dorner and Hellinger procedure were positive by the quick microtechnique.

*M38. Further Observations on the Occurrence of Streptococci Other Than Group A in Human Infection.* GEORGE E. FOLEY, The Children's Hospital and Infant's Hospital, Department of Pathology, Boston, Mass.

During a 2-year period the streptococci isolated from 118 cases of suppurative or generalized streptococcal infection were classified as to serological group. These cases represent but a small percentage of the total streptococcal admissions to the institutions where these data were collected during this time, and were selected in that only infections thought likely to be nonrespiratory in origin were studied. Non-group-A streptococci were isolated from 95 (80.0 per cent) of these cases. Groups D, B, K, C-G, F, and E were encountered in that order of frequency. Seventy-two (75.0 per cent) of these non-group-A strains occurred as pure cultures on primary isolation. The predominance of group D streptococci could be accounted for in part by the number of cases of urinary infections and subacute bacterial endocarditis in the series. However, even if these cases (50) were omitted, group D was second only to group A in frequency of occurrence. Of 34 cases of subacute bacterial endocarditis, group D streptococci were isolated from 19 (55.9 per cent), group K from 1 (2.9 per cent), and serologically unclassified alpha strains from 14 (41.2 per cent). Of the latter group, 6 were *Streptococcus s.b.e.*, 5 were *Streptococcus bovis*, and 3 were *Streptococcus milis*. Alpha and gamma as well as beta strains were encountered among these non-group-A streptococci. Consideration of the epidemiology of non-group-A streptococci suggests endogenous rather than exogenous events precipitating infection.

M39. *The Action of Pasteurella pestis Bacteriophage on Pasteurella, Salmonella, and Shigella.* A. S. LAZARUS AND J. B. GUNNISON, University of California Medical School, Department of Bacteriology, San Francisco 22, Calif.

A strain of *Pasteurella pestis* phage, which lysed all of 12 strains of *P. pestis* studied, has been investigated to determine its ability to lyse other bacteria. Solid and liquid media were used. Nineteen out of 27 strains of *Pasteurella pseudotuberculosis* were lysed by the phage. After adaptation to *P. pseudotuberculosis*, the phage lysed all strains of that organism, most of them in higher dilution than originally. Three of 42 *Salmonella* strains and six out of 37 *Shigella* cultures were susceptible to *P. pestis* phage. Seventy-seven cultures from 17 other genera were not affected by the same phage. After adaptation to *Shigella* species, the phage showed an increased potency toward the six susceptible *Shigella* strains. After adaptation to *Shigella* or *P. pseudotuberculosis*, the phage still retained completely its ability to lyse *P. pestis*. Minor serological relationships were shown to exist between *P. pestis* and certain strains of *Salmonella* and *Shigella*, using macroscopic agglutination tests. These relationships were not clearly correlated with susceptibility to phage. Either phage action in this case is not based on surface antigens held in common, or the minor antigenic relationship shown to exist between *P. pestis* and some salmonellas and shigellas is an unstable one. The use of *P. pestis* lysates as vaccines and the study of the lethal properties of some *P. pseudotuberculosis* lysates are suggested as worthy of further investigation.

M40. *The Effect of Yeast Concentrate on the Growth and Survival of Hemophilus influenzae in Infusion Broth.* ERWIN NETER, University of Buffalo and Children's Hospital, Department of Bacteriology and Immunology, Buffalo, N. Y.

Since it was shown that yeast concentrate supported the growth of *Neisseria gonorrhoeae*, experiments were carried out to determine whether it also enhanced the growth and survival of *Hemophilus influenzae*. Several strains of *H. influenzae* type b, freshly isolated from patients with meningitis, and several stock culture strains of types a, b, and c (maintained on chocolate agar) were seeded into brain heart infusion broth containing various concentrations of yeast concentrate (bacto-supplement B, Difco Laboratories) as well as into broth without concentrate. The culture media were incubated at 37 C. The resulting visible growth was noted and subcultures were made, after various periods of incubation, to chocolate agar. In contrast to the control broth, visible growth may occur within 24 to 48 hours in broth containing yeast concentrate in concentrations ranging from 1 per cent to 20 per cent. Higher concentrations (5 per cent to 20 per cent) were found to be more effective than lower concentration (1 per cent). In infusion broth containing the yeast concentrate, *H. influenzae* type b produces SSS, as demonstrated by precipitation tests with type b-specific anti-*H. influenzae* serum. In the absence of grossly visible growth

yeast concentrate definitely prolonged the survival of *H. influenzae* in infusion broth. It is concluded that an autolysate rich in coenzyme, glutamine, co-carboxylase, and other B-vitamin factors favorably influences the growth of *H. influenzae* in infusion broth. The possible practical applications of this finding will be discussed.

*M41. A Simplified Liquid Culture Medium for the Growth of Hemophilus pertussis.* W. F. VERWEY, ELIZABETH H. THIELE, AND DOROTHY N. SAGE, Sharpe & Dohme, Inc., Department of Bacteriology, Glenolden, Pa.

The liquid culture medium described by Hornibrook has been simplified and modified by the removal of calcium chloride, sodium chloride, and sodium carbonate, the rebalancing of the quantities of casein hydrolyzate, potassium phosphate, and nicotinic acid, and the substitution of cystine for cystine hydrochloride. All ingredients are stable and readily available and may be added prior to sterilization. Fourteen strains of freshly isolated *Hemophilus pertussis* have been found to grow readily in this medium, and two have been carried through 19 consecutive subcultures without loss of virulence or evidences of phase changes. Cultures grown in this medium are not granular and develop to a density of 30 to 40 billion cells per ml in 48 hours. Vaccines prepared from liquid cultures have been found to be equal or superior to those prepared from organisms grown on Bordet-Gengou agar on the basis of mouse protection tests using the intracerebral route of infection. The liquid medium can be prepared as an agar containing 5 per cent human blood cells and this has been found to produce more colonies from suspensions of *H. pertussis* than does Bordet-Gengou agar. Because of the simplicity and growth-promoting qualities of this agar medium, its use as a diagnostic medium is suggested.

*M42. An Analysis of Weckstein's Rapid Method for the Primary Identification of the Gonococcus.* J. D. THAYER, MATTHEW A. BUCCA, AND RUTH A. KIRTY, Research Laboratory, U. S. Public Health Service, New York (Staten Island 4), N. Y.

In a study of Weckstein's method for isolation and identification of gonococci a variety of irregular, atypical, and misleading color reactions were found on the glucose and maltose plates. Forty-three per cent of the cultures showed typical fermentative color characteristics of the gonococcus. Twenty-three per cent of the cultures produced red-colored gonococcus colonies on both glucose and maltose plates and would have falsely indicated the meningococcus or *Neisseria sicca*, *Neisseria flava*, *Neisseria perflava*, or *Neisseria subflava*. Twenty-three per cent of the specimens did not grow on either the glucose or maltose plate and would have resulted in false negative reports. The highest concentration of the dye that can be added to the medium so that typical gonococcic fermentation color reactions are observed, varies considerably. The presence of contaminating bacteria can adversely influence the fermentative color reaction of the gonococcus colonies.

*M43. The Effect of the Cyclic Changes of the Cervical Mucus upon the Isolation of the Gonococcus from Cervical Cultures.* MARIE L. KOCH, Johns Hopkins Medical School, Department of Bacteriology, Baltimore, Md.

The use of pancreatic digest agar containing 5 per cent human chocolate blood should have eliminated the cultural difficulties encountered in isolating gonococci from cervical cultures. This, however, was not the case, so further studies were made to determine what other factor, or factors, were responsible for obtaining negative cervical cultures in cases of clinical gonorrheal cervicitis. In 1940 Lamar, Shettles, and Delfs showed that there was a relative change in the alkalinity and acidity of the cervical mucus during the menstrual cycle, and that there was a positive correlation between these cyclic changes with the penetrability of spermatozoa. Investigations were made to determine whether or not these cyclic changes had any effect upon the viability of the gonococci *in vivo*. A study of 50 untreated dispensary patients and 5 hospitalized patients with a clinical diagnosis of gonorrheal cervicitis showed that negative cervical cultures are associated with acid mucus and that only negative cultures are obtained during the latter part of the luteal phase of the cycle when the pH range of the cervical mucus is 6.6 to 5.2. Positive cultures are associated with the estrogenic phases of the cycle when the pH range of the cervical mucus is 7.6 to 6.8. There is a positive correlation between the cyclic changes in the pH of the cervical mucus and the ability to isolate gonococcus from cervical cultures.

*M44. The Selection of a Suitable Medium for Culturing Root Canals.* DONALD E. SHAY, University of Maryland Dental School, Department of Bacteriology, Baltimore, Md.

It is possible that some of the fermentative changes involved in the production of acid on the enamel surface of the tooth occur under both anaerobic and aerobic conditions. Thus, a medium most suitable for culturing root canals should promote growth of both types of organisms. Trypticase glucose broth and a modified thioglycolate medium were selected as the experimental media. As controls, four of the most frequently used media were chosen: brain heart infusion, Blayney's brain agar, Brewer's thioglycolate, and serum broth. Sterile tubes containing 2 ml of nutrient broth and alundum were inoculated with a paper point which had been in the root canal from 1 to 2 minutes. To obtain a suspension of the organisms present, the paper point was ground against the alundum with a sterile 1-ml pipette. Aseptically, 0.1 ml of the nutrient broth was added to each medium and incubated for 2 weeks. Slides were prepared from all negative tubes. If positive growth resulted, it was streaked to blood agar plates and incubated for 24 hours. Slides were then prepared from both the plate and the tube. Of the 709 root canals cultured, 183 were positive on one or more of the various media. Using trypticase glucose broth (pH 7.2), 166 were positive; 158 using brain heart infusion; 144 using serum broth; 137 using Blayney's brain agar; 131 using Brewer's thioglycolate; and 95 using trypti-

case glucose broth (pH 5.5). Modified thioglycolate was discontinued after checking 203 root canals. Of the 183 positive cultures, 84 were caused by the presence of gamma streptococci, 44 by staphylococci, and 20 by a combination of organisms. The trypticase glucose (pH 7.2) proved to be the most satisfactory medium, yielding a higher percentage of positives in the shortest period of time.

*M45. The Poisson Series in the Evaluation of Media Used for the Detection of Shigellae and Salmonellae.* OSCAR FELSENFELD, Mount Sinai Medical Research Foundation, Chicago 8, Ill.

It was observed that the distribution of plates streaked with 2 to 7 shigellae or salmonellae follows the Poisson series. To utilize this observation in the evaluation of diagnostic media, series of 100 plates each of 20 media were inoculated with suspensions of fastidious *Shigella dysenteriae* or *Salmonella sendai* and a "contaminating mixture." The true number of organisms in the suspension was determined by plating to tryptose agar. The means, the class frequencies, and the goodness of fit of the examined and of the control plates were compared. A satisfactory probability of detecting *S. dysenteriae* could be expected only by streaking series of 5 to 6 reliable plates (MacConkey, Levine, Panja and Ghosh, desoxycholate, D. C. L. S.). The use of tetrathionate broth or selenite enrichment with consecutive streaking of two media (S. S., D. C. L. S., or Panja and Ghosh) increased the probability of the isolation of *S. sendai* to 0.99. When similar series of plates were tested with less fastidious, but in America more frequent, organisms (*Shigella paradysenteriae* III and *Eberithella typhosa*) with a combination of one little selective plate (MacConkey, Levine, desoxycholate, D. C. L. S.), two medium selective plates (S. S. or D. C.), and one enrichment tube (tetrathionate or selenite) streaked to a bismuth sulfite plate (Wilson and Blair, Hajna and Perry, or Difco), the probability of a positive result was higher than 0.97.

*M46. Relative Productivity of Newer Coliform Media.* ELSIE WATTIE, U. S. Public Health Service, Department of Water and Sanitation Investigations, Cincinnati 2, Ohio.

The newer media for the detection of bacteria of the coliform group in water, which are reported to have given promising results, are lauryl sulfate tryptone broth (L.S.T.) by Mallman and *Escherichia coli* broth (E.C.) by Perry. These two media have been compared with standard lactose using water samples of widely varying quality. Presumptive tubes of all three media were incubated at 37 C. In addition duplicate sets of tubes of the E.C. medium were incubated at 44 C and 45.5 C in both air and water immersion incubators. All tubes showing gas formation in any amount were confirmed in brilliant green bile broth and on eosin methylene blue agar plates. Cultures which confirmed were purified and subjected to the completed tests. Detailed data on the productivity of these media will be presented. In general it may be said: (1) Standard

lactose broth produced a considerable number of false presumptives. (2) L.S.T. broth was almost as productive as standard lactose broth, the number of false positives were reduced, but results were unreliable without confirmation. (3) E.C. medium at 37 C was less productive than the other media, with fewer false positives. (4) The productivity of E.C. medium at 45.5 C in both air and water immersion was less than 50 per cent of that of the other two media or of E.C. at 37 C. (5) E.C. medium at 44 C was slightly more productive than at 45.5 C.

*M47. Studies on Rabies Infection in Developing Chick Embryos.* HILARY KOPROWSKI AND HERALD R. COX, Lederle Laboratories Division, American Cyanamid Company, Section of Viral and Rickettsial Research, Pearl River, N. Y.

The chick-brain-adapted Flurry strain of rabies was used. Egg passages were initiated with a 138th-chick-passage brain suspension. The virus was carried through 40 passages by inoculation into the yolk of fertile hens' eggs. Subsequent experiments showed that inoculation into the allantoic sac gave equally good results. The LD<sub>50</sub> titers of embryos infected by the yolk sac route ranged from 10<sup>-5</sup> to 10<sup>-6</sup>. The virus content of the embryo was highest between the 7th and 12th days after inoculation, the incubation period being inversely proportional to the concentration of virus in the inoculum. The virus was present in all tissues. The virus content was greatest in the embryo itself, the concentration in the CNS being only slightly higher than in the remainder of the embryo. Embryonic blood is infectious from the 3rd to 15th day after inoculation. All embryos inoculated on 7, 9, 11, and 13 days of incubation died at time of hatching. Embryos inoculated on 15, 17, and 19 days of incubation ordinarily hatched out, but some died soon after hatching with virus recoverable from their brains. Surviving chicks possessed high titer neutralizing antibodies in their sera obtained 14 days after hatching. The chick-embryo-adapted virus is pathogenic for mice, guinea pigs, hamsters, and cotton rats inoculated intracerebrally. It was of low virulence for rabbits intracerebrally and avirulent parenterally. The living virus protected mice against intracerebral challenge with Pasteur strain rabbit virus and protected rabbits against parenteral challenge with Pasteur strain guinea pig virus.

*M48. Transmission of Human Poliomyelitis Virus to Mice.* ALBERT MILZER, CHESTER L. BYRD, AND SIDNEY O. LEVINSON, Michael Reese Research Foundation, Michael Reese Hospital, Department of Bacteriology, Chicago, Ill.

We have previously reported that autolyzed brain diluent shortens the incubation period and facilitates the transfer of poliomyelitis virus to CFW Swiss mice, hamsters, and rhesus monkeys. The Leon monkey passage strain of poliomyelitis virus has been successfully adapted to CFW mice by means of this technique. In the present studies poliomyelitis virus was isolated in CFW Swiss mice from 11 to 12 stools obtained during the acute stage from patients



with paralytic poliomyelitis involving two or more extremities. Poliomyelitis virus was also isolated in mice from spinal cord from a fatal case of bulbar poliomyelitis. Autolyzed brain tissue diluent was prepared as described previously. The proper preparation of the autolyzed brain is of critical importance for successful transmission. Stool suspensions were prepared by grinding feces in approximately 10 volumes of sterile distilled water, centrifuging at 2,000 rpm for 10 minutes, and treating the supernatant after chilling with ether (20 per cent by volume) overnight in the refrigerator. On the following day, the ether was evaporated by negative pressure, and the suspension was centrifuged at 5,000 rpm for 1 hour. The supernatant was next removed, mixed with equal volumes of autolyzed brain, and inoculated intracerebrally and intraperitoneally into CFW mice. Proof of adaptation was shown by successful transfer to rhesus monkeys and neutralization by 1:50 dilution of human serum immune globulin.

*M49. Studies of the Distribution of Poliomyelitis Virus. IV. In Rural Schools Following an Epidemic.* THOMAS FRANCIS, JR., AND GORDON C. BROWN, School of Public Health, University of Michigan, Department of Epidemiology, Ann Arbor, Mich.

In August, 1945, the rural schools in Henderson County, Tennessee, were opened toward the end of an epidemic of poliomyelitis in that area. In view of the much debated question of opening schools following epidemics a study was undertaken to reveal evidence of the occurrence and possible spread of the virus under such circumstances. Stools were collected from all 99 pupils of two rural schools on the opening day and again 2 weeks later. Collections were also made at the same times from 37 children in the small town of Lexington, whose school was not to open for a month. The stools were pooled in groups of three, rendered bacteriologically sterile, and inoculated into monkeys. If positive, the pool was broken down and the specimens tested separately. All the specimens from one school were negative on the opening day, but 2 weeks later one was positive. Two children in the second rural school were carrying the virus on the opening day but only one of these was positive 2 weeks later. All other tests were negative at both collections. In the control group, one was positive at the first collection but all were negative two weeks later. The results indicate that although these children may have undergone a previous subclinical infection, few were found to be carriers of poliomyelitis at the time of the study. Though virus was present on the opening day, there was no greater spread among the pupils than in children not yet returned to school.

*M50. Laboratory Studies on the Epidemiology of Poliomyelitis.* F. B. GORDON, F. M. SCHABEL, JR., ALBERT E. CASEY, AND WILLIAM I. FISHBEIN, University of Chicago, Department of Bacteriology and Parasitology, and the Chicago Health Department, Chicago, Ill.

During an epidemicologic study of poliomyelitis in Chicago in 1945, stool specimens were obtained from children classified as household contacts, non-

household contacts, and noncontacts, residing in the neighborhood of paralytic cases. Control specimens were also obtained in nonpoliomyelitis neighborhoods. Tests of stool specimens of 71 children by combined intracerebral, intraperitoneal, and intranasal inoculation of monkeys revealed virus in the following incidences: of 20 household contacts, 16 (80 per cent) were positive; of 28 non-household contacts, 10 (36 per cent) were positive; of 18 noncontacts, 2 (11 per cent) were positive; of 5 controls, none was positive. The average age of the children in the four groups was similar in each case. Of the total of 28 children with virus in their stools, only 3 had clinically recognizable poliomyelitis. These results indicate a high incidence of subclinical infection in the immediate contacts of poliomyelitis cases, and provide added evidence for transmission of the virus by direct contact.

*M51. Active Immunity in Monkeys to Poliomyelitis Virus.* ISABEL M. MORGAN, Poliomyelitis Research Center, Johns Hopkins University, Department of Epidemiology, Baltimore 5, Md.

Immunity to intracerebral or intranasal inoculation of Lansing poliomyelitis virus has been induced in *M. rhesus* monkeys by intramuscular injection of active Lansing virus. This immunity is associated with high titers of serum antibody. Monkeys were vaccinated intramuscularly with 20 per cent Lansing infected monkey spinal cord. Six weeks after receiving total doses of virus suspension ranging from 0.8 g to 3.2 g infected spinal cord given in 4 injections, all of 17 monkeys resisted intracerebral injection of 10 per cent Lansing monkey cord suspension. This represented 10,000 PD<sub>50</sub>. The group was then divided. One-half proved immune to subsequent intranasal instillation of active Lansing virus, whereas the other half became paralyzed after a similar instillation of Brunhilde virus. This heterologous strain has been shown to have only slight immunological relationship with Lansing virus. Serum neutralization tests were carried out with Lansing virus in mice. The 50 per cent end points of the sera of immunized monkeys just prior to intracerebral challenge ranged from 1/1,000 to 1/8,000. Thus by intramuscular vaccination with active virus monkeys have been rendered solidly immune to intracerebral or intranasal inoculation of homologous poliomyelitis virus. This immunity is associated with high titers of circulating antibody. These monkeys failed to resist intranasal instillation of a heterologous strain of poliomyelitis virus.

*M52. The Antibody Response in Human Beings Inoculated with Japanese Encephalitis Vaccine.* JOEL WARREN, JOSEPH E. SMADEL, AND A. F. RASMUSSEN, JR., Army Medical Department Research and Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

Previous experiments have shown that Japanese encephalitis vaccine prepared from infected chick embryos immunized mice against infection as well as did mouse brain vaccine. The serological response of human beings to chick

embryo and mouse brain types of Japanese encephalitis vaccine was studied. In addition, the response following different methods of administration was tested. Administration of two doses of 2 ml at a 4-day interval elicited demonstrable neutralizing antibody in about 30 per cent of the persons who received either chick embryo or mouse brain type vaccine. When chick embryo type vaccine was given in 1-ml amounts on 1, 7, and 30 days, approximately 60 per cent developed neutralizing substances. In contrast, complement-fixing antibodies of Japanese encephalitis appeared rarely and in low concentration. Six persons who had neutralizing antibodies following immunization according to the second schedule were bled at 5 months. Only two still possessed immune substances, and these maintained them for a year. Reinjection of four individuals with 1 ml of chick embryo vaccine 1 year after the basic immunization elicited neutralizing antibodies in those who had shown antibody following the basic immunization.

*M53. Complement-fixing Antibodies Reacting with Normal Chick Embryo Antigens in Sera of Persons Repeatedly Immunized with Chick Embryo Type Vaccines.* JOSEPH E. SMADEL, JOEL WARREN, AND MERRILL J. SNYDER, Army Medical Department Research and Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

In measuring the response of human beings to Japanese encephalitis vaccine, chick embryo type, 72 soldiers were selected who had previously been immunized with vaccines prepared in embryonated eggs (yellow fever, typhus, influenza). Thirty-eight volunteers received a course of two injections of chick vaccine and 34 received three injections. They were bled before vaccination and after the second and third injections. Sera were tested for complement-fixing antibodies against normal and infected chick embryo antigens, which were clarified by centrifugation at 12,000 rpm. Sera from 21 of the 72 persons reacted with normal embryo antigen prior to the first injection of encephalitis vaccine. Fifty-five of the 72 soldiers gave positive reactions after two injections, and of 34 given a third dose 31 were positive. The complement-fixation titers ranged from  $\frac{1}{2}$  to  $\frac{1}{32}$  in prevaccination sera and covered the same range in the post-vaccination specimens. Four of twelve positive reactors still had antibodies, titers  $\frac{1}{2}$  to  $\frac{1}{16}$ , 5 months after vaccination. Fourteen of the higher titered sera were tested for Wassermann-reacting substance by the Kolmer technique; all were negative. The substance which reacted with the sera of vaccinated individuals occurred in greater amounts in antigens made from 13-day embryos than from 6-day embryos. Ten soldiers who possessed antibody prior to vaccination were devoid of cutaneous sensitivity to chick embryo antigen. No untoward allergic reactions occurred in this group of 72 individuals as a result of injection of vaccine.

*M54. Vaccination Against Q Fever.* MERRILL J. SNYDER, JOSEPH E. SMADEL, AND FREDERICK C. ROBBINS, Army Medical Department Research and

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*M54. Vaccination Against Q Fever.* MERRILL J. SNYDER, JOSEPH E. SMADEL, AND FREDERICK C. ROBBINS, Army Medical Department Research and

Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

Formalinized vaccines prepared from rodent or yolk sac tissues infected with *Rickettsia burneti* have been shown by others to induce resistance in animals to infection with this agent. Ten per cent suspensions of yolk sacs infected with the Henzerling (Italian) and Dyer (American) strains were inactivated with formalin and extracted with ether. Such vaccines injected subcutaneously or intraperitoneally into guinea pigs in 1 to 3 doses of 1 ml each elicited appreciable amounts of complement-fixing antibody which reacted with Henzerling antigen but not with Dyer antigen. Beginning about 30 days after vaccination, antibodies against Dyer antigen appeared and increased in titer until they approached the Henzerling level at 60 days. Guinea pigs immunized with either type vaccine displayed resistance to infection with both strains. The vaccines protected guinea pigs against death from Q fever and mitigated the febrile response, but did not induce complete resistance to very large doses of *R. burneti*. Thirty-two persons were immunized with 3 subcutaneous injections of 1 ml of vaccine. Twenty-one of the 25 receiving Henzerling vaccine developed complement-fixing antibodies with titers ranging from  $\frac{1}{2}$  to  $\frac{1}{32}$ , average  $\frac{1}{16}$ . Only 1 of the 7 receiving Dyer vaccine developed antibodies. Sera of all 22 persons who developed antibodies reacted with Henzerling antigen but only 1 of these fixed with Dyer antigen. No data became available on the resistance of vaccinated persons to infection. The variations in serological response will be discussed.

*M55. Metabolic Differences Between Phage-susceptible and Phage-resistant Variants of a Strain of Escherichia coli.* HENRY W. SCHERP, S. FARNUM COFFIN, AND JOHN F. WALDO, University of Rochester School of Medicine and Dentistry, Department of Bacteriology, Rochester 7, N. Y.

The dependence of bacteriophage upon the bacterial host for reproduction suggested that the acquisition of resistance to bacteriophage by the host organism might be accompanied by changes in metabolic activities. Resistant variants were isolated from lysed cultures of a strain of *Escherichia coli* susceptible to Burnet's bacteriophage C13. By the usual cultural tests, these variants were identical with the parent strain, except that indole was not produced and they grew more slowly. Methylene blue reduction times were determined simultaneously for the resistant and susceptible organisms at 37 C under vaseline seal in mixtures comprising washed organisms (0.015 mg bacterial nitrogen per ml), 1:250,000 methylene blue, M/40 phosphate buffer at pH 7.4, and 0.0001 M substrate. The average ratios, reduction time for resistant : reduction time for susceptible, were: glucose, 1.2; glycerol, 2.6; lactate, 4.1; succinate, 3.8; pyruvate, 1.4; alanine, 2.6; galactose, 1.9; maltose, 1.4. Under the conditions of these experiments, methylene blue was not reduced in the presence of acetate, citrate,  $\alpha$ -ketoglutarate, glutamate, tyrosine, xylose, lactose, and dulcitol. Infection of susceptible cells with the phage produced no significant change in the

methylene blue reduction times unless the reaction was allowed to progress to the verge of lysis, whereupon all dehydrogenase activity disappeared. The results showed that the acquisition of resistance to the bacteriophage was paralleled by a diminution of various metabolic activities of the host organism. No evidence regarding the specificity of these findings was provided.

*M56. Reactivation of Ultraviolet-inactivated Bacteriophage Particles Inside Double-infected Host Cells.* S. E. LURIA, Indiana University, Bacteriological Laboratories, Bloomington, Ind.

Inactivation of a bacteriophage particle by ultraviolet radiation can be detected by the inability of the particle to produce more phage when absorbed by a sensitive bacterial cell. A large amount of reactivation takes place, however, if concentrated suspensions of inactivated phage are put in contact with sensitive bacteria. Statistical analysis proves that reactivation occurs in a fixed proportion of those bacterial cells that absorb two inactive phage particles. The probability of reactivation is a function of the dose of radiation received by the bacteriophage. Reactivation only occurs for "large particle" phages. Cross reactivation can also take place between particles of serologically related phages, not between unrelated strains. No reactivation was found after X-ray inactivation. By analogy with the phenomenon of transfer of genetic characters between phage particles growing in the same host cell, the reactivation experiments are tentatively interpreted as indicating genetic transfers at loci that have undergone "lethal" mutations. Statistical analysis of the experimental data proves them compatible with this hypothesis. The results are analyzed in their bearing on the genetic constitution of the phage particle and its growth mechanism.

*M57. In Vitro Responses of Actinomyces bovis to Sulfonamides and Antibiotics.* MILAN NOVAK AND THELMA FLANDERS, University of Illinois, Department of Bacteriology and Public Health, Chicago, Ill.

The present study was done to test the effect of streptomycin, penicillin, and sulfa drugs on five strains of *Actinomyces bovis*, three of human and two of bovine origin. Strain susceptibility has been ascertained and the relative amounts of tested agents required for inhibition determined. Chick embryos were infected with this organism but the results, after using chemotherapeutic agents, were not conclusive because of inherent difficulties in this method. The experimental work utilized thioglycolate media, into which was incorporated varying concentrations of the test drugs. The antagonistic activity of this medium against the drugs is taken into consideration, as are also the difficulties in interpretation of the results caused by the cultural idiosyncrasies of the organism. Under the conditions of these experiments it was found that for three human and two bovine strains sulfathiazole and sulfadiazine were not inhibitory in concentrations of 50 mg per cent; penicillin was inhibitory in concentrations varying from 0.1 to 0.5 units per ml; streptomycin inhibited all but one strain

in concentrations of 100 units per ml. From these results penicillin and streptomycin appear to be more effective against *Actinomyces bovis* than the sulfonamides in clinically practical concentrations.

*M58. In Vivo Studies on the Effect of BAL on the Trypanocidal Activity of Arsenicals.* WILLIAM WILSON AND N. ERCOLI, Warner Institute for Therapeutic Research, Department of Pharmacology and Chemotherapy, New York, N. Y.

The purpose of this investigation was to determine the influence of BAL on the therapeutic activity of arsenicals in relation to its detoxifying effect. Mice infected with *Trypanosoma equiperdum* were treated subcutaneously with various amounts of BAL in oil and with mapharsen, which were injected at separate sites. BAL interfered with the therapeutic effect of mapharsen in doses much lower than those required to antagonize toxic mapharsen doses. (Lethal doses of mapharsen required 2 treatments with 60 to 100 mg per kg BAL in order to obtain survival.) Two treatments with BAL (5.0 mg per kg) completely abolished the trypanocidal activity of the curative dose of mapharsen by single injection (5.0 mg per kg). With lower doses of BAL (1.25 to 2.5 mg per kg) relapses followed and BAL doses still lower than these (0.6 mg per kg) did not influence noticeably the therapeutic activity. A higher dosage of mapharsen, such as 20 mg per kg, required for inhibition 15 to 10 mg per kg BAL. Correspondingly, the minimal dose of arsenical required for therapy increased in proportion to the amount of BAL administered. It is concluded that the loss of toxicity of the arsenical induced by BAL is accompanied by a corresponding, or even greater, loss in the therapeutic effectiveness.

*M59. Streptomycin in the Treatment of Experimental Trypanosomiasis in White Mice and Chick Embryos.* DONALD J. MERCHANT AND M. H. SOULE, University of Michigan, Department of Bacteriology, Ann Arbor, Mich.

White mice and chick embryos were infected with *Trypanosoma brucei*, *Trypanosoma equiperdum*, and *Trypanosoma hippicum*. After a lapse of 24 to 36 hours streptomycin hydrochloride was administered subcutaneously to the mice and was injected into the yolk sac of the chick embryos. Each mouse received a total of 16,000 units and each chick embryo a total of 40,000 units of streptomycin. The course of the disease was followed by microscopic examination of blood specimens taken at regular intervals. As far as could be determined, this antibiotic agent did not alter the course of the infections or prolong the life of the treated mice or embryos.

*M60. Streptomycin in Experimental Brucellosis.* E. H. KELLY AND THOMAS F. HENLEY, JR., Department of Research and Development, Camp Detrick, Frederick, Md.

The effect of streptomycin on experimental brucellosis was investigated in mice and guinea pigs. Mice infected with *Brucella suis* and treated with strep-



tomycin from the time of infection were sacrificed in two groups, half at the end of therapy and the remainder 2 weeks later. The first group showed a lower incidence of infection than the controls but in the latter group the number showing infection was higher than in the untreated control group. Guinea pigs infected with *Brucella suis* in a dose insufficient to infect all of the controls responded to streptomycin therapy. In animals receiving a heavier infecting dose (approximately 24 ID<sub>50</sub>) no significant effect of the antibiotic could be detected. It appears, therefore, that streptomycin, in the form in which it was used, is of little value for the therapy of brucellosis.

*M61. Comparative Effectiveness of Penicillins F, G, K, and X in Spirochetal Infections as Determined by Short in Vivo Methods.* THOMAS B. TURNER, MARY C. CUMBERLAND, AND HUAN-YING LI, Johns Hopkins School of Hygiene and Public Health, Department of Bacteriology, Baltimore, Md.

A method has been developed for the *in vivo* assay of various penicillins against *Treponema pallidum*. The assay requires 3 weeks and results are qualitatively similar to those obtained by the only other *in vivo* method yet reported, one which requires large numbers of rabbits and 9 months for completion. Multiple syphilomas are produced on rabbits' backs by intracutaneous inoculation. Spirochete counts are made on representative lesions before, and 24 hours after, treatment with penicillin, which is given in three equal doses at 2-hour intervals. Pretreatment counts averaged about 1,000 *T. pallida* per 200 dark fields. To reduce the count to 10 or fewer spirochetes in 50 per cent of the animals required 0.1 mg per kg of penicillin G, 0.6 mg per kg of F, 1.1 mg per kg of X, and more than 2.0 mg per kg of K. Assays have also been made against the spirochete of relapsing fever, *Borrelia novyi*. Mice are treated 24 hours after infection and counts made 24 hours later. Controls show about 27 spirochetes per 3-minute count. To reduce the count to two or fewer spirochetes in 50 per cent of the mice requires 8.3 mg per kg of penicillin G, 15 mg per kg of F, 24 mg per kg of X, and 37 mg per kg of K. While this test seems less sensitive than that for *T. pallidum*, the results show the same qualitative differences between the penicillins. Results obtained by these short methods are consistent and apparently reflect within limits the comparative therapeutic activity of these four penicillins against experimental syphilis and relapsing fever.

*M62. A Morphological Variant of Escherichia coli and Its Resistance to Streptomycin.* ESTHER STUBBLEFIELD, The Upjohn Company, Department of Bacteriological Research, Kalamazoo, Mich.

During a study of acquired resistance of bacteria to streptomycin an atypical form of *Escherichia coli*, possibly identical to Kuhn's A forms, was encountered. This organism when transferred from streptomycin agar to plain agar grew only as globular cells varying in diameter from 1 to 7 microns. On streptomycin agar it grew as a mixture of bizarre rods with branching and curved forms and a

few round cells. Repeated transfers of the variant on plain agar brought about a gradual reversion to the typical rod form of *E. coli*. Alternate transfers on plain agar and streptomycin agar maintained the culture in the round form. This atypical form of *E. coli* became resistant to 10,000 micrograms of streptomycin per ml of agar much more slowly than did 11 other gram-negative rods and 8 colony subcultures of the same strain of *E. coli*. When a resistant culture of the globular organism was transferred on plain agar until the culture consisted of predominantly typical rod forms, the only cells which retained resistance to the high level of streptomycin were the few atypical forms remaining. Cultural and antigenic studies were made on the globular culture in comparison with the parent strain of *E. coli*.

*M63. Anaphylaxis in the Fish.* N. B. DREYER AND J. W. KING, University of Vermont, Medical College, Departments of Pharmacology and Bacteriology, Burlington, Vt.

A series of teleost fish was sensitized to various antigens in an attempt to demonstrate the production of anaphylaxis in these cold-blooded vertebrates. The antigen was given intraperitoneally both for the sensitizing and for the subsequent shocking dose. In all instances sensitivity could be demonstrated after 11 days and after periods as long as 6 weeks. An injection of antigens at intervals after the first shocking reaction demonstrated a repetition of anaphylaxislike symptoms previously observed. The use of antigens on normal fish and saline on sensitized fish showed the reaction to be a specific one. A similar but more violent and transitory phenomenon could be produced using relatively large doses of histamine. Following injection of the shocking material the fish showed moderate uneasiness as manifest by swimming agitatedly about the jar and bumping its nose on the sides of the container. Subsequent to this one could observe a fanning of the dorsal fin, most marked in the anterior part. There was also an associated curling and closing of the caudal fin and a temporary loss of equilibrium. As these symptoms subsided the fish fell to the bottom of the jar and lay quietly for some time. There was marked increase in the excursions of the gill clefts, which may or may not have been accompanied by an increase in respiratory rate. The reaction has never been lethal although only minute doses of shocking antigen have been used.

*M64. Effects of Antihistaminic Substances on the Tuberculin Reaction.* JORGEN M. BIRKELAND AND LOTTIE KORNFELD, Ohio State University, Department of Bacteriology, Columbus, Ohio.

There is no general agreement as to the role of hypersensitivity in the development of resistance to infection in tuberculosis, nor is there any agreement as to the nature of the tuberculin reaction. It is generally assumed that histamine or histaminelike substances are involved in the hypersensitive state. In an attempt to determine whether these agents play a part in the immune response and in the tuberculin reaction, infected animals were treated with antihistaminic agents, and the effect on the tuberculin reaction as well as on the course of the

infection as shown by survival time was observed. Rabbits and guinea pigs were immunized to histamine azo-protein ("hapamine") and then infected. Other groups of animals were infected and treated with benadryl and pyribenzamine. Tuberculin sensitivity and survival time were determined. The results indicate that the antihistaminic agents employed did not delay the appearance of the tuberculin reaction, lessen its severity, nor affect the longevity of the animals. The question is raised as to whether the hypersensitive manifestations of tuberculosis involved histamine or histaminelike substances. Since antihistaminic agents ordinarily successful in combating anaphylactic and allergic phenomena failed to show a significant effect on the tuberculin reaction, these data cast doubt on the assumption that histamine or histaminelike substances play a predominant role in the reaction in rabbits and guinea pigs.

*M65. The Application of the Ascoli Test in Tularemia.* CARL L. LARSON, National Institute of Health, Division of Infectious Diseases, Bethesda, Md.

The Ascoli test was studied employing suspensions in 0.85 per cent salt solution of cultures of *Pasteurella tularensis* and of spleen and liver tissue from animals dying of tularemic infections. The suspensions were heated for 30 minutes in flowing steam in the autoclave, cooled, centrifuged, and the clear supernatant fluid was employed as antigen. Immune serums from humans convalescent from tularemia and certain other diseases and serums from immunized animals were employed in the tests. Precipitin tests were done by the capillary tube and macroscopic methods. The antigen reacted with serums from cases of tularemia but failed to react with serums from cases of brucellosis, typhus fever, Rocky Mountain spotted fever, typhoid fever, and shigellosis. Tularemia serums reacted with antigens prepared from *P. tularensis* but not with antigens prepared from *Brucella* sp., *Pasteurella septica*, *Shigella dysenteriae*, and *psittacosis* virus. No precipitation occurred with normal serums. White mice, white rats, and guinea pigs were infected with *P. tularensis*. The spleens and livers were removed 24 hours after death and portions were converted to Ascoli antigen and tested. The remainder was placed in the incubator at 37 C and at intervals of 7 and 14 days further tests were performed. There was no significant loss of antigenicity during this period. The antigen was precipitated by acetone. The soluble antigen prepared by ether extraction of organisms yielded Ascoli antigen when heated. Adaptation of the Ascoli test to tularemia has been of value in establishing early diagnosis and may be of practical value in ecological studies of tularemia.

*M66. Studies on the Pathogenesis and Immunity in Tularemia. I. The Course of Infection with Bacterium tularense as Seen in the Normal, Vaccinated, and Recovered White Rat.* CORA M. DOWNS, LUTHER BUCHELE, AND BARBARA J. OWEN, University of Kansas, Department of Bacteriology, Lawrence, Kans.

The first step in the study of the pathogenesis of tularemia was accomplished

by the following experiments. Normal, vaccinated, and recovered rats were used in groups of 40 each. The rats were injected with multiple infective doses of a virulent strain of *Bacterium tularensis* by the intraperitoneal, subcutaneous, or intradermal routes. Two animals from the normal, vaccinated, and recovered groups were bled for antibody determination, killed, and quantitative plate counts were made on the blood and weighed pieces of tissue. Animals were killed immediately after inoculation, at 12 hours, daily for 4 to 5 days, then at weekly intervals. Half of the animals were used for statistical results on mortality. It is apparent that in the normal animals the organisms invaded the blood stream within a few hours and persisted in large numbers until death. In other tissues there was multiplication of the organisms within 24 hours, increasing to the 3rd or 4th days, the days of greatest mortality in the normal infected animals. By the 5th day any normal surviving rats were convalescent and the organisms were decreasing in number. In the vaccinated and recovered animals the organisms were first found in the regional lymph nodes, in the spleen, and in other tissues, but in fewer numbers than in normal animals. In the convalescent animal the organisms disappear from the tissues in the following order, blood, liver, lymph nodes, and spleen. They may persist in the spleen for 50 days after infection.

*M67. Quantitative Aspects of Streptomycin Therapy in Experimental Tularemia.*

JOSEPH T. TAMURA AND WILLIAM SUYEMOTO, University of Cincinnati, College of Medicine, Department of Bacteriology, Cincinnati, Ohio.

Preliminary *in vitro* tests in a gelatin hydrolyzate liquid medium, inoculated to give 2 million cells per ml, gave bacteriostatic levels of 0.2 to 0.4 microgram per ml. Visible turbidity during 7 days of incubation was the criterion for growth. When viability was judged similarly after periodic subcultivation to fresh medium, the bactericidal values per exposure periods were 1.0 microgram per ml for 30 minutes, 2 micrograms per ml for 8 minutes, 4 micrograms per ml for 2 minutes, and 6 micrograms per ml for less than 1 minute. The median effective dose of streptomycin was determined for white mice after subcutaneous challenge with 30 to 50 LD<sub>50</sub> doses of a strain of maximal virulence. One-quarter of the total streptomycin, calculated for mice in each group of a twofold geometric dosage series, was injected intraperitoneally immediately after challenge, and the remainder in 3 equal doses at intervals of 2 hours. Eight tests and titrations gave ED<sub>50</sub> doses from 352 to 358 micrograms. Hence the average ED<sub>50</sub> dose for these arbitrary and convenient conditions of challenge and treatment was 350 micrograms of streptomycin per 20-gram mouse, or 17.5 micrograms per gram.

*M68. Universal Serological Reactions with Lipid Antigen in Leprosy.* REUBEN

L. KAHN, FLORA T. VILLALON, AND BETTY J. BARIBEAU, University Hospital, Serology Laboratory, Ann Arbor, Mich.

It was observed that quantitative precipitation systems with sera and lipid antigens employing salt concentrations lower than physiological (0, 0.15, 0.3,

0.6 per cent) and higher than physiological (1.2, 1.5, 1.8, 2.5 per cent) will give positive reactions after icebox incubation in practically all persons and in animals. In some instances, universal reactions are also obtained on immediate readings of the tests, without incubation. The term "universal" has been applied to these reactions because they apparently represent a common characteristic of all sera. The strength, and serological patterns, of these reactions vary to some extent in different persons and in different animals. The present report deals with results of universal reactions in cases of leprosy. It was found that in tuberculoid leprosy, in which the host's immunity to the disease is presumably high, the precipitation results, without incubation, are practically negative. In lepromatous leprosy, in which the host's immunity to the disease is presumably low, the precipitation results, without incubation, are markedly high. The precipitation results in transitional cases of leprosy are intermediate between these two extremes. It is believed that these findings are of practical value in the diagnosis of the various types of leprosy and in the prognosis of the disease.

*M69. A Practical Method of Pertussis Vaccine Assay by Mouse Protective Test.*  
AUGUST HOLM, E. R. Squibb and Sons, Biological Laboratories, New Brunswick, N. J.

There is no uniformly accepted method of determining the antigenicity of *Hemophilus pertussis* vaccine. The method described here uses Swiss mice, which are given four subcutaneous injections of decreasing vaccine dilutions at an interval of respectively 2, 3, and 4 days, followed by an intraperitoneal challenge dose in mucin suspension 10 days after the last immunizing injection. By using a series of decreasing vaccine amounts for the immunization of the mice, it is possible to determine the LD<sub>50</sub> point of a certain vaccine, and consequently to assay its antigenicity quantitatively. The test is easy to perform and has given satisfactory results in our hands for several years.

*M70. Specific Aggregation of Streptococcal Proteins Adsorbed on Oil Globules.*  
*II. Behavior of Acid-precipitable Fraction.* D. A. BOROFF AND L. M. TRIPP, JR., Camp Detrick, Frederick, Md.

A bacterial antigen in solution may possess several specificities, some of which become apparent only under special conditions. Adsorption of a group-specific protein (NPA) derived from streptococci, Lancefield group A, on olive oil changes the reactivity in an agglutination reaction from predominantly group-specific to predominantly type-specific. When this protein is adsorbed on olive oil globules, it behaves serologically and immunologically more like intact streptococci than when it is in solution. NPA adsorbed on olive oil globules induces the production of type-specific antibodies when injected into rabbits, shows a prozone in agglutination reactions in the region of antibody excess, and exhibits cross reactivity in low dilutions of antisera. Adsorption of NPA on a non-streptococcal bacterial surface does not change the specificity of the protein from

that exhibited by NPA in solution. It is suggested that the type-specific activity of NPA complex may be due to Lancefield's substance "T."

*M71. Persistence of Antigen at the Site of Inoculation of Vaccine Emulsified in Oil.*

MIRIAM HERDEGEN, SEYMOUR P. HALBERT, AND STUART MUDD, University of Pennsylvania, Department of Bacteriology, Philadelphia, Pa.

It has been postulated that persistence of antigen in mineral oil emulsion at the site of inoculation permits opportunity for hyperimmunization by the slow, continuous absorption of antigen from the localized mass of vaccine, accounting in part for the adjuvant effect of mineral oil on vaccines. The present work was undertaken to study the persistence of antigen quantitatively, as determined by the ability of the residual antigen removed from the original test animal to stimulate antibody production in other mice to which the antigen was transferred. The correlation between the residual antigen and antibody titer in original test mice was also determined. The results indicate that active antigen persisted at the site of inoculation up to 18 and 24 weeks, depending on the original dose of antigen, but there was a gradual diminution with time in the amount recovered. Only a relatively small amount of this deterioration of antigen at the site of inoculation was paralleled by a fall in the agglutinin titers of the mice receiving the vaccine. The data presented afford quantitative evidence that, for the system studied, prolonged absorption of active antigen is a prime factor involved in the adjuvant effect of the mineral oil menstruum.

*M72. Antibody Response to Alkali-soluble Protein Extracts of Corynebacterium diphtheriae and Derived Variants.* H. E. LIND, Sias Laboratories, Brookline, Mass.

An attempt was made to determine precipitin production by alkali-soluble protein extract of *Corynebacterium diphtheriae*, several derived variants, and *Corynebacterium hofmanni*. The alkali-soluble protein extracts were prepared according to the method of Wong and T'ung (1939). Significant amounts of nitrogen were obtained from the virulent parent strain, approximately 10 times that of the nonvirulent parent type variants and about 50 times that of the nonvirulent small colony variants. Superimmunization of rabbits was necessary in order to produce high titers. The antiprotein serum from the parent strain showed precipitation not only with the homologous protein extract but with extracts of parent type variants. It exhibits no precipitation with the small nonvirulent variants. The parent type variants showed a low degree of cross reaction with the parent antiserum suggesting alteration in composition or reduction of protein. In the small variants the protein appeared altered beyond detection or destroyed, there being no cross reaction with the parent antiserum. The quantity of protein in the extract was directly correlated with the strain of *C. diphtheriae* that maintained virulence, there being considerably less in the nonvirulent variants and these also being less capable of producing antibody formation when injected into rabbits.

*M73. Immunization of Mice Against Pneumococcal Pneumonia by Inhaled Polysaccharide.* VERNON BRYSON AND MARYDA SWANSTROM, Biological Laboratory, Cold Spring Harbor, N. Y.

As part of a research program conducted for the Medical Division, Chemical Warfare Service, an attempt was made to immunize mice via the respiratory route against experimental pneumonia. Eighty CFl female mice weighing about 20 grams were divided in four lots of 20 animals. Twenty of these animals were set aside as controls. The remaining 60 mice were exposed in glass chambers to aerosolized polysaccharide in three equal groups, receiving respectively 1, 5, and 25 milligrams of type I polysaccharide generated as aerosol by nebulization of 3 ml of antigen solution in 90 liters of air during a 15-minute period. The 15-minute exposure to polysaccharide aerosol was repeated on the third and sixth days following initial exposure to antigen. Six days after the final exposure to polysaccharide all animals, including controls, were injected intraperitoneally with approximately  $4 \times 10^4$  type I pneumococci suspended in 0.1 ml of sterile broth. On the basis of experiments conducted with radioactive tracers it may be estimated that 0.3 per cent of the dispensed polysaccharide was retained in each animal under conditions of the experiment. Total polysaccharide dispensed and estimated individual dose inhaled may then be correlated with mortality: Group I, untreated; no survivors in twenty. Group II, estimated polysaccharide inhaled per animal, 9  $\mu$ g; one survivor in twenty. Group III, estimated polysaccharide inhaled per animal, 45  $\mu$ g; two survivors in twenty. Group IV, estimated polysaccharide inhaled per animal, 225  $\mu$ g; thirteen survivors in twenty. Immunity against pneumococcal infection by exposure to polysaccharide aerosol has almost certainly been produced in some animals of group IV ( $P = <.01$ ).

*M74. Antigens of Vegetable Origin Active in Pneumococcus Infections.* LLOYD D. FELTON, BENJAMIN PRESCOTT, GLADYS KAUFFMANN, AND BARBARA OTTINGER, National Institute of Health, Division of Infectious Diseases, Bethesda, Md.

This investigation was motivated by the fact that bacteria are plantlike organisms, and consequently have certain biological characteristics in common with members of the vegetable kingdom. Both bacteria and plants contain polysaccharides, water-soluble at pH 7.0, and classified in the latter as hemi-, or pseudo-, or reserve cellulose. The immediate objective was to determine whether these plant polysaccharides have antigenic properties similar to those of bacteria, especially pneumococci. Thus far 59 members of the vegetable kingdom have been studied. Soluble products were obtained with definite immunological activity as measured by both precipitin tests and active immunity in mice against virulent pneumococci. Analysis of the active fractions, with two exceptions, showed the presence of hydrolyzable polysaccharides with sugar content ranging from 5 to 49 per cent. Both viscosity and optical rotation were measured. However, the degree of correlation was low between these results and the degree of antigenicity. Although of relatively low titer as measured by precipitin reactions, most of these samples stimulated active immunity in mice

against from 100 to 1,000,000 lethal doses of virulent pneumococci. Fractions from collard, Irish moss, sunflower seed, tomato, and wheat germ were of this maximum titer. In some cases a single preparation produced active immunity against three types of pneumococci, types I, II, and III, the only types thus far studied. Preliminary tests indicate that this polyvalent characteristic may be due to a mixture of type-specific components. In no instance were precipitins or active immunity of as high titer as those of the antigenic polysaccharide of the pneumococcus secured. It has been observed that 0.01  $\mu$ g increases resistance of mice to the degree that they survive at least 1,000 lethal doses of virulent pneumococci.

*M75. An Analysis of Rabbit Mortality and Plasma Potency in Production of Anti-Hemophilus influenzae Type B Rabbit Serum.* A. W. TALLMAN, F. D. EATON, AND E. G. GERWE, E. R. Squibb & Sons, Bacteriological Production Laboratories, New Brunswick, N. J.

For economical and efficient large volume production of anti-*Hemophilus influenzae* type B rabbit serum, it is important to be able to predict the mortality and expected yield from a given number of animals. These predictions are aided by collection and analysis of data from a large number of hyperimmunized rabbits which were subject to specified production procedures. Rabbits were started on a schedule of hyperimmunization in groups of 200, and the group mortalities were recorded from day to day for more than 10,000 rabbits. This procedure permitted construction of three mortality curves from which it is possible to predict the volumes of blood obtainable at various periods of immunization, depending upon the time at which the first production bleedings are taken. Since the greatest mortality occurs during the weeks that production bleedings are taken, it is important to correlate mortality and potency in order to determine the most economical time at which to take the first production bleedings. A tabulation has been prepared which correlates potency and mortality studies, and the data indicate that, within the specifications of the production methods employed, it is most economical to take first production bleedings during the eleventh week of hyperimmunization instead of the fifth or eighth week, and the total yield of antibody units is relatively the same whether first production bleedings are taken on the fifth, eighth, or eleventh week.

*M76. Studies on the Effect of Immune Reactions on the Respiration of Bacteria.*  
*I. Methods and Results with Eberthella typhosa.* M. G. SEVAG AND RUTH E. MILLER, University of Pennsylvania, Department of Bacteriology, and Woman's Medical College of Pennsylvania, Philadelphia, Pa.

The effect of homologous immune serum with and without complement on the oxygen consumption by *Eberthella typhosa* (strains O-901 and H-901) and pneumococcus has been studied. A method has been worked out which makes it possible to calculate  $QO_2$  values ( $\text{mm}^3 O_2$  per mg bacteria per hour) for intact, ag-



glutinated, and lysed fractions of bacteria. Agglutinated *E. typhosa* and pneumococci consume volumes of oxygen equal to those of the respective controls. Apparently in intact sensitized bacterial cells, the activity of the intracellular enzymes is not affected by this reaction. Sensitized *E. typhosa* (O-901) cells acted upon by complement undergo lysis, eliminating the cell wall barriers to the action of the immune factors on intracellular enzymes. Immediately after lysis the liberated enzymes use considerably more oxygen than the controls containing the intact cells. Subsequently, the oxygen consumption of the lysed system undergoes up to 88 per cent reduction. In systems containing unlysed bacteria, oxygen consumption in the presence of glycerol alone or yeast extract and glucose is markedly greater than when glucose alone is used. Under these conditions, the reduction in oxygen uptake of the lysed systems is likewise much greater and prompter. Potassium cyanide causes 90 per cent inhibition of oxygen consumption by the unlysed and lysed cells, showing that the oxygen consumption in lysed systems is mediated by intact and oxidative enzymes.

*M77. Contribution to B.C.G.: Experiments on Bovines.* ALBERTO A. ASCOLI, Cornell Medical College, Department of Public Health and Preventive Medicine, New York City, N. Y.

The Instituto Vaccinogeno Antituberculare provided the first definite proof that B.C.G. protected the bovine species against natural infection through exposure and that a reduction of the disease to a half could be obtained by means of annual revaccinations. However, even when given its best chance, B.C.G. failed to provide complete protection to calves which were confronted with uninterrupted exposure for 10 months offered by facing cows affected with open tuberculosis. Better results can hardly be expected from the cross immunization one has to deal with, when B.C.G., an attenuated bovine strain, is being administered to human beings as a prophylactic against infection with the human type of *Mycobacterium tuberculosis*. Attention is called to the influence of other extrinsic factors, mainly age, on the development of the specific resistance. Under the circumstances you are bound to question whether, in their anxiety to ensure its harmlessness, Calmette and Guérin did not go too far in the attenuation of the virulent bovine strain; if so, it is understandable why the peak of immunity developed by the B.C.G. might not reach at all the level giving complete protection. In such a case a stronger vaccine, that is, a less attenuated strain, ought to be resorted to. Actually several less attenuated strains have been prepared by the IVA and tested on bovines for their harmlessness and efficiency.

*M78. Streptomycin Dosage Schedules.* ERNA ALTURE-WERBER AND LEO LOEWE, Jewish Hospital, Department of Laboratories, Brooklyn, N. Y.

Streptomycin blood levels were assayed following fractional, intramuscular administration of the antibiotic. The method of assay, previously described

by the authors, was made sensitive by utilizing *Klebsiella pneumoniae* as the test organism. With this method, detectable amounts of streptomycin were found for 24 to 36 hours following a single intramuscular injection of 250 to 1,000 micrograms. Optimum dosages were predicated on *in vitro* streptomycin sensitivity tests of the infective organisms. Although both bacteriostatic and minimal lethal dosages were estimated, clinical dosage schedules were based on the latter. The peak levels after 500 milligrams were suitable for many streptomycin inhibitable organisms, and endured for at least 6 hours. A rational method for planning the intermittent, intramuscular injection was thus established.

**M79. *The in Vitro Streptomycin Sensitivity of Salmonella Isolated from Cases and Carriers in Massachusetts.*** GEORGE E. FOLEY AND A. DANIEL RUBENSTEIN, The Children's Hospital and Infants' Hospital, and Massachusetts Department of Public Health, Department of Pathology, Boston, Mass.

Seven strains of *Eberthella typhosa* and 57 strains of *Salmonella*, covering 15 species, all recently isolated from cases and carriers in Massachusetts, have been studied for *in vitro* sensitivity by titration with varying concentrations of streptomycin in tryptic digest broth. The species examined were *S. typhi-murium* (24), *S. thompson* (6), *S. paratyphi* B (5), *S. newport* (4), *S. enteritidis* (3), *S. montevideo* (3), *S. minnesota* (2), *S. st. paul* (2), *S. tennessee* (2), and one strain each of *S. derby*, *S. manhattan*, *S. morbilliformis-bovis*, *S. newington*, *S. oregon*, and *S. oranienberg*. All strains were inhibited by streptomycin concentrations ranging from 0.004 to 0.064  $\mu\text{g}$  per ml. Although there seemed to be significant differences in the sensitivity of individual strains of the same or different species, no species was more susceptible or resistant than other species. In general, strains isolated from related cases or carriers in different areas of Massachusetts exhibited similar *in vitro* sensitivity. The range of *in vitro* streptomycin sensitivity of freshly isolated salmonellas is essentially similar to that previously observed in a survey of a collection of stock strains.

**M80. *Prolongation of Penicillin Activity in Animals.*** ROGER D. REID, Hynson, Westcott & Dunning, Inc., Biological Research Division, Baltimore 1, Md.

*In vitro* studies have disclosed a few substances that have the ability to protect penicillin from the action of penicillinase and penicillinaselike enzymes. These studies have been continued with special reference to prolongation of penicillin levels in serum and enhanced protection of infected animals given penicillin in conjunction with such compounds. This report will summarize these investigations and will survey the possible therapeutic application of the compounds studied.

**M81. *A Preliminary Report on a Selective Medium for the Isolation of Pathogenic Fungi.*** PAUL J. BOEING AND NORMAN C. LAFFER, Bowey's, Inc.,

Chicago, Ill., and University of Maryland, Department of Bacteriology, College Park, Md.

The mycology section of this laboratory was confronted with a suspected case of maduromycosis, in which much secondary infection was evidenced. It was decided to try the medium suggested by Thompson (1945), which utilizes 10 units of streptomycin per ml and 2 units per ml of penicillin in an attempt to isolate the causative organism. Thompson's medium was tried in the manner suggested in his publication and the infectious material, curetted from the lesions of the suspected maduromycosis, streaked on the plates. The secondary bacterial invader grew in abundance, so it was decided that higher concentrations of the antibiotics mentioned should be utilized. To determine the optimum antibiotic concentrations necessary, several series of media were prepared using increasing amounts of streptomycin and penicillin. The infectious material from the suspected maduromycosis, *Blastomyces dermatitidis*, *Candida albicans*, *Sporotrichum schenkii*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Monosporium apiospermum*, *Blastomyces brasiliensis*, and several strains of gram-negative and gram-positive bacteria were inoculated onto these plates. One lot of media was prepared using no antibiotics at all as a control. The information presented in this paper indicated that a medium containing heart infusion agar base with 6 per cent human blood, 25 units of streptomycin, and 6 units of penicillin per ml should be satisfactory for the isolation of pathogenic fungi from infectious material.

*M82. Studies on the Causal Agent of Granuloma Inguinale.* R. B. DIENST, C. R. REINSTEIN, H. S. KUPPERMAN, AND R. B. GREENBLATT, University of Georgia School of Medicine, Department of Medical Microbiology and Public Health and Endocrinology, Augusta, Ga.

The present investigation presents method used to isolate and cultivate Donovan bodies from patients with granuloma inguinale. Preliminary report is given on reproducing clinical symptoms in human volunteers using pure cultures of organisms grown in yolk sac of developing chick embryo.

*M83. Morning Versus Evening Rectal Temperature Response in Rabbits.* H. E. WRIGHT AND I. B. DORRELL, Schenley Laboratories, Inc., Physiological Control Laboratory, Lawrenceburg, Ind.

This investigation was undertaken to determine whether rabbits could be used for pyrogen testing at night as well as during the day. Control rectal temperatures of ten male New Zealand rabbits in the weight range of 2,200 to 2,700 grams were taken and recorded in the morning and again in the evening, 5 days a week for 6 weeks. The animals were housed in an air-conditioned and fluorescent-lighted environment throughout the test. Pyrogen tests were run on the same rabbits both in the morning and at night 5 days a week for 2 weeks. There was only a slight increase in evening rectal temperature response over morning temperature response in both the pretest and pyrogen-testing periods. All

temperatures remained within the normal range as prescribed by the U. S. P. XII. There was no significant change in the weight of the animals during the 6-week control period nor during the 2-week pyrogen-testing period. These data seem to indicate that rabbits can be used for pyrogen testing at night as well as during the day. Further studies with actual pyrogenic stock are contemplated using rabbits as test animals both in the morning and at night.

*M84. Cutaneous Reactions in Persons Suffering from Diverse Diseases Following Intradermal Injection of Streptococcal Antibody and Antigen.* EDWARD C. ROSENOW, Longview Hospital, Bacteriologic Research Laboratory, Cincinnati, Ohio.

The study concerns the isolation of specific types of alpha streptococci, and production and intradermal use of natural and artificial antibody and of antigen to determine the presence of respective specific streptococcal antigen and antibody in the skin or blood of persons ill. Pure cultures of specific types of streptococci were obtained from the end point of growth of serial dilution cultures in glucose brain broth of material obtained from nasopharynx, tonsils, or infected teeth. The centrifugated organisms from large volumes of glucose broth were preserved in dense suspensions in two parts glycerol and one part saturated NaCl solution.

Horses were immunized and artificial antibody obtained, using antigen prepared from appropriate dilutions of these dense suspensions. Ten per cent solutions of the euglobulin fraction of the serum of immunized horses and the supernatant of suspensions of 10 billion streptococci per ml that had been autoclaved for 96 hours were used as natural and as artificial antibody in cutaneous tests for the detection of antigen and the supernatant of corresponding suspensions heated to 70 C for one hour was used for the detection of antibody in skin or blood. Cutaneous reactions indicating the presence of specific antigen were consistently obtained with natural and artificial antibody at all stages of the disease, often proportional to its severity, and specific antibody during convalescence in persons suffering from influenza, other respiratory infections, "virus" pneumonia, encephalitis, poliomyelitis, arthritis, epilepsy, and schizophrenia. Reactions in well persons tested as controls did not occur or were relatively slight.

*M85. Differences in Strains of Rickettsia orientalis as Demonstrated by Cross-Vaccination Studies.* FRED L. RIGHTS, JOSEPH E. SMADEL, AND ELIZABETH B. JACKSON, Army Medical Department Research and Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

Scrub typhus vaccines were prepared from lungs and spleens of white rats infected with Imphal, Karp, Kostival, or Mite 21 strain of *Rickettsia orientalis* and assayed by methods previously described. Groups of mice were immunized with these four vaccines and tested for resistance to infection, with the

homologous strain. In addition, groups of mice immunized with each vaccine were challenged with one of seven heterologous strains of the organism. Most of the vaccines protected against 1,000 to 10,000 MLD of the homologous strain. In practically all instances greater protection was elicited against the homologous than against the heterologous organisms. All four types induced immunity to Imphal, Karp, and Kostival rickettsiae. Mite 21 vaccine provided protection against these three strains, but vaccines prepared from the latter failed to protect against Mite 21. None of the four vaccines immunized mice against the Seerangayee strain. Some but not all of the vaccines protected against Wild Rat 235, Volner, and Pescadores strains. Thus, strain differences in *R. orientalis* are demonstrated by cross-vaccination studies as well as by studies employing neutralization, complement-fixation, and antitoxin tests. Despite these demonstrable differences, mice recovered from infection with any one of the strains studied are solidly immune to challenge with the heterologous strains. No correlation existed between the antigenic relationships of the strains and their geographical origins. The need for a broadly antigenic strain for use in preparing vaccine for immunization of human beings will be discussed.

*M86. Differences in Strains of Rickettsia orientalis as Demonstrated by Cross-Neutralization Tests.* BYRON L. BENNETT, JOSEPH E. SMADEL, AND ROSS L. GAULD, Army Medical Department Research and Graduate School, Department of Virus and Rickettsial Diseases, Washington 12, D. C.

Previous work performed in this laboratory indicated that differences in strains of *Rickettsia orientalis* were demonstrable by means of cross-neutralization, cross-vaccination, and cross-antitoxin tests. Bengtson has also demonstrated differences in strains of this organism by complement-fixation tests. Ten strains of *R. orientalis* recovered from man, mites, or rodents from widely scattered areas in the South Pacific and Orient were used to prepare antisera in rabbits and to perform neutralization tests with these sera in mice. Pooled immune rabbit sera protected against the homologous strain in each instance but provided variable protection when tested against the other nine heterologous strains. Antisera against certain of the strains, i.e., Seerangayee and Gilliam, protected little if any against infection with the heterologous strains. On the other hand, Volner and Buie antisera contained substances which provided at least some protection against practically all of the heterologous organisms. Present information is insufficient to arrange an immunological pattern but does indicate that certain strains occupy an intermediate position between those which are very broad and those which are very narrow. Furthermore, the data are consistent with findings obtained by other techniques which indicate that antigenic variations exist among strains now grouped under *R. orientalis*.

*M87. The Practical Application of an Oiling Program in the Control of Respiratory Disease.* I. L. SHECHMEISTER AND FRANCIS S. GREENSPAN, University

of California, Department of Bacteriology, Berkeley, Calif., and The New York Hospital, New York, N. Y.

This study, carried out under the auspices of the U. S. Navy, dealt with the role of oiled floors and blankets in control of certain respiratory diseases. The group chosen for the investigation consisted of approximately 2,400 men, who were divided equally into an experimental and a control group. The floors of the barracks housing the experimental group were treated with a 20 per cent germicidal oil-water-roccal emulsion, while the blankets were impregnated with 2 to 3 per cent (by weight) of a similar preparation. Periodic determinations were made of the bacterial content of dust, air, and blankets, as well as of the dispensary admission rates and the carrier rates for beta hemolytic streptococci. The results indicated that the above treatment of floors and blankets (1) reduced the number of organisms in the air 33 to 63 per cent; (2) sharply reduced the total amount of dust in the oiled environment, although it apparently did not change the beta hemolytic streptococcus count per gram of dust—isolated streptococci were mostly nonpathogenic, only 5 per cent being typed in group "A"—(3) caused a slight but significant reduction in the beta hemolytic streptococcus carrier rates in the group living in oiled environs; (4) seemed to cause a reduction in the number of cases of respiratory disease during periods of low respiratory disease incidence, but had no effect during a period of high respiratory disease incidence.

*M88. Production of Anti-Hemophilus influenzae Type B Rabbit Serum.* F. D. EATON, E. G. GERWE, AND G. F. LEONARD, E. R. Squibb & Sons, Bacteriological Production Laboratories, New Brunswick, N. J.

Preliminary laboratory procedures involving the use of relatively small groups of rabbits had indicated that the titer of the plasma of hyperimmunized rabbits reached a level of 0.4 to 0.6 mg of agglutinin nitrogen per ml by the fifth week. In order to determine some of the factors which contributed to yields of relatively low potency plasma when a large scale production schedule was followed, an analysis is made of data from over 1,000 rabbits used in production of anti-*Hemophilus influenzae* type B rabbit serum. From the production data a potency curve is drawn, from which it is possible to follow the average titer of production groups at various periods of immunization. This curve indicates that the average rise in titer is much slower in large groups of rabbits than is the case with individually selected animals which, by small groups, are the subjects of assiduous laboratory experiments. For the large groups of animals, it is profitable to omit the fifth and eighth week bleedings. The plasma volumes obtained from the groups at various periods of immunization are given, and by means of correlation of these volumes and the potency curve it is found that the average potency of combined plasma from all bleedings over a period of 20 weeks is 0.30 mg of antibody nitrogen per ml. When refined and concentrated, this plasma yields a final product which contains 3.0 mg of antibody nitrogen per ml.

*M89. Localization of Radioactive Azoprotein in Tissues.* HERBERT J. WELSH-  
IMER, GRANT L. STAHLY, AND WILLIAM G. MYERS, The Ohio State  
University, Department of Bacteriology and Medicine, Columbus 10,  
Ohio.

*Para*-aminobenzenesulfonic acid labeled with radioactive antimony was coupled with purified egg albumin by diazotization. Following the injection of this substance the localization of a defined protein in the animal body was observed by sacrificing the animals, removing the various tissues, and determining their radioactivity by means of a Geiger-Müller counter.





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# THE ANTIGENICITY OF CRYSTALLINE LYSOZYME

JOSEPH SMOLENS AND JESSE CHARNEY<sup>1</sup>

*Wyeth Institute of Applied Biochemistry, Philadelphia, Pennsylvania*

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The first attempt to produce lysozyme antiserum seems to have been that of Jermoljewa and Bujanowskaja (1931). Unpurified lysozyme was used. Roberts (1937) described results obtained with a purified but not crystalline lysozyme preparation. He reported that lysozyme is antigenic, that precipitins are formed, and that rabbit antiserum inhibits lysozyme activity on *Micrococcus lysodeikticus*. Fleming and Allison (1922) reported that serum had the property of lysing *M. lysodeikticus*. Roberts did not mention whether this characteristic of serum interfered with his inhibition tests. The following communication gives the results of experiments in which recrystallized (4 to 6 times) egg white lysozyme was utilized. The lysing property of normal serum was taken into consideration.

## MATERIALS AND METHODS

*Antigen.* Two lysozyme preparations from egg white were used in this study. Both were crystalline. One was prepared by the method of Alderton *et al.* (1945) and was recrystallized six times. The other was prepared by the method of Alderton and Fevold (1946) and was recrystallized four times.

*Sera.* Antisera were prepared in chinchilla rabbits either by repeated intravenous injection, or by subcutaneous injection of a water in oil emulsion according to the method of Freund and McDermott (1942).

*Test for lysozyme activity.* A suspension of *M. lysodeikticus* was distributed into a number of vials and dried from the frozen state. For each test a fresh vial was opened and planted on two nutrient agar slants. These were incubated at 37 C for 16 hours and the growth was washed off with 0.85 per cent NaCl and centrifuged. The sediment was suspended in 0.85 per cent NaCl to give a turbidity reading of 100 on the Klett-Summerson photoelectric colorimeter using a no. 42 filter. One ml of this living suspension was added to 1-ml volume of test solution, and the tubes were placed at 37 C. Readings were taken at varying time intervals; for the purpose of this study, readings at 4½ hours were selected as most suitable, as they gave optimal lysis and eliminated potential complication due to contaminants.

*Inhibition of lysozyme with specific antiserum.* To the varying concentrations of crystalline lysozyme in 0.5-ml volume were added 0.5 ml of varying dilutions of serum. The tubes were shaken, placed at 37 C for 1 hour, and then to each mixture 1 ml of *M. lysodeikticus* suspension (described under test for lysozyme activity) was added. The tubes were shaken and placed at 37 C, and the extent of lysis was recorded at ½, 1, 2, 3, 4, and 5 hours.

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*Precipitin test.* These tests were made by mixing 0.4 ml of antigen in the proper dilution with 0.4 ml of undiluted antiserum. The tubes were placed at 37 C for 1½ hours, transferred to the refrigerator overnight, and the amount of precipitate was estimated after centrifugation.

#### EXPERIMENTAL RESULTS

*Lysozyme activity of normal rabbit serum.* In corroboration of Fleming's original observation (1932) it was found that rabbit sera exhibited varying degrees of

TABLE 1  
*Normal rabbit serum lysozyme activity*  
(4.5-hour readings)

RABBIT	FINAL SERUM DILUTION		
	1:6.7	1:67	1:670
S-1	+	±	0
S-2	0	0	0
S-3	+	±	0
S-4	+	±	0
S-5	±	0	0
S-6	±	0	0
S-7	±	±	0
S-8	+	±	0
S-9	+	±	0
S-10	+	±	0
S-33	±	±	0
S-0	+	±	0
S-1011	+	±	0
S-170	+	±	0
S-177	+	±	+
S-182	+	±	0
S-1477	+	±	0
S-1479	+	±	0
S-1974	+	±	0
S-196	±	±	0

0 = no lysis; ± = partial lysis; + = complete lysis.

lytic activity against *M. lysodeikticus*. One hundred and three normal rabbits were bled from the marginal ear vein; the serum was separated and tested for lytic activity. Three dilutions of serum each in a volume of 0.3 ml were employed (0.3, 0.03, and 0.003 ml), the diluent being extract broth. To the contents of each tube, 0.7 ml of broth and 1 ml of *M. lysodeikticus* suspension (described under Materials and Methods) were added, and the tubes were shaken, placed in the 37 C incubator for 4½ hours, and read. The degree of resulting lysis was recorded. The results of a representative number of such tests are shown in table 1.

Rabbit serum S-2 seemed to have no lytic activity. Three rabbits out of a

total of 103 were found to be in this category. It is interesting that this lytic property of serum seems to be inherent in the eight species of animals tested including humans. The role of the active constituent and the reason for its absence in a small percentage of rabbit sera are obscure.

*Preparation of antilysozyme sera in rabbits.* A series of 12 intravenous injections (total of 17 mg of crystalline lysozyme each) were made in two rabbits. No

TABLE 2

*Rabbit antibody response to combined intravenous lysozyme immunization and saline in oil emulsion*

SERA	FINAL ANTIGEN DILUTION					
	1:10 <sup>2</sup>	1:4 × 10 <sup>3</sup>	1:16 × 10 <sup>3</sup>	1:64 × 10 <sup>3</sup>	1:256 × 10 <sup>3</sup>	1:1024 × 10 <sup>3</sup>
51	0	0	0	0	0	0
52	0	±	1	4	4	1
NRS	0	0	0	0	0	0

4 = large amount of precipitate with perfectly clear supernatant; 3, 2, 1 = decreasing amounts of precipitate with decreasing clarity of supernatant; ± = trace; 0 = no precipitate; NRS = normal rabbit serum (control).

TABLE 3

*Comparison of precipitin response with the lytic activity of serum*

SERA	PRECIPITIN TITER			LYSOZYME ACTIVITY			
	Final Lysozyme Dilution			Final Serum Dilution			
	1:50 × 10 <sup>3</sup>	1:200 × 10 <sup>3</sup>	1:800 × 10 <sup>3</sup>	1:6.6	1:66	1:666	1:6,666
½	tr	tr	1	±	0	0	0
12	—	3	2	±	0	0	0
S-2	4	0	0	0	0	0	0
101	—	0	0	+	0	0	0
112	4	4	3	+	±	0	0
104	0	1	1	+	±	0	0
108	0	0	0	+	±	0	0
109	4	1	0	+	0	0	0
111	0	2	1	±	0	0	0
NRS	0	0	0	±	0	0	0

0 = no lysis; ± = partial lysis; + = complete lysis; tr = trace.

precipitating antibodies were observed in sera obtained at varying time intervals. A water in oil emulsion containing 15 mg of lysozyme was then prepared and injected subcutaneously into one of the two rabbits (no. 52). The other rabbit (no. 51) received 6 more intravenous injections totaling 18 mg of lysozyme. One week later both rabbits were bled (interval of 3 weeks for no. 51 rabbit) and precipitin tests made. The results are shown in table 2.

Another series of four rabbits received 15 intravenous injections totaling 35 mg of lysozyme each. Only 1 of the 4 sera gave a positive precipitin titer, this

being active to a lysozyme dilution of 1:200,000. A further series of eight rabbits were injected subcutaneously with a total of 15 mg of lysozyme in water in oil

TABLE 4  
*Inhibition of lysozyme activity by specific rabbit antisera*

FINAL LYSOZYME DILUTION	TIME OF READING <i>hours</i>	ML SERUM /TUBE							
		0.1	0.05	0.025	0.012	0.006	0.003	0.0015	0
1:40 × 10 <sup>3</sup>	$\frac{1}{2}$	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+
1:400 × 10 <sup>3</sup>	$\frac{1}{2}$	0	0	0	0	+	+	+	+
	1	0	0	0	+	+	+	+	+
	2	±	0	0	±	+	+	+	+
	3	±	±	0	+	+	+	+	+
	4	±	±	±	+	+	+	+	+
	5	±	±	±	+	+	+	+	+
1:4,000 × 10 <sup>3</sup>	$\frac{1}{2}$	0	0	0	0	0	0	0	+
	1	0	0	0	0	0	0	0	+
	2	±	0	0	0	0	0	0	+
	3	±	±	±	0	0	0	0	+
	4	±	±	±	±	0	0	0	+
	5	±	±	±	±	0	0	0	+
1:40,000 × 10 <sup>3</sup>	$\frac{1}{2}$	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0
	2	±	0	0	0	0	0	0	0
	3	±	±	±	0	0	0	0	±
	4	±	±	±	±	0	0	0	±
	5	±	±	±	±	0	0	0	+
Control, no lysozyme	$\frac{1}{2}$	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0
	2	±	±	0	0	0	0	0	0
	3	±	±	±	±	0	0	0	0
	4	±	±	±	±	0	0	0	0
	5	±	±	±	±	±	0	0	0

0 = no lysis (inhibition of lysozyme); ± = partial lysis (partial inhibition); + = complete lysis (no inhibition).

*Note:* A precipitate appeared in a few tubes in the 1:40 × 10<sup>3</sup> dilution of lysozyme which was due to combination with the antibody. This precipitate was easily distinguishable from the tubes with growth present.

emulsion. These rabbits were bled 4 weeks later, and 5 out of 8 rabbits were found to have developed precipitins in their sera.



The results obtained on the small number of rabbits in these experiments would appear to indicate that crystalline lysozyme is antigenic and that the antibody production may be increased by the use of "falba" and mineral oil as adjuvants. The antigenicity of lysozyme is certainly not so marked as that of a typical protein. Actually much difficulty was encountered in the production of antisera. Whether this is due to the presence *in vivo* of a substance which inactivates the injected lysozyme or whether lysozyme is absorbed and utilized rapidly, thus decreasing its antigenic efficacy, is not known. It is clear that the body temperature does not cause inactivation since the serum lytic factor is resistant to relatively high temperatures, i.e., 56 to 60 C for days.

*Attempt to correlate loss of lytic activity of rabbit serum with the appearance of antibody to crystalline lysozyme.* It was thought that with the production of antibody to crystalline lysozyme there might be a fall in the lytic activity of the rabbit serum. Table 3 shows that there is apparently no correlation between precipitin titers and lysozymic titers of various sera. This result is one that might have been anticipated since data are available showing that enzymes, prepared from different sources but having similar substrate activities, demonstrate entirely different immunological specificities. Also, of course, antibodies to a normally present constituent would be an unexpected phenomenon, although this has been reported when adjuvants have been utilized (Kopeloff and Kopeloff, 1944).

*Inhibition of crystalline lysozyme activity by specific rabbit antisera.* The procedure used is described under methods. Table 4 shows that antilysozyme rabbit serum definitely inhibits the lysozyme activity. It is interesting to note how the control series with no egg lysozyme shows lysis of the substrate in the lower serum dilutions. Here again differences in immunological specificity are clearly demonstrated, that is, despite the two lysozymes having apparently similar lytic activities, antiserum to one displays no inhibiting effect on the other lysozyme.

#### DISCUSSION

The results of the present study show that crystalline egg white lysozyme is antigenic. This enzyme stimulates the formation of specific antibodies when injected into some rabbits and reacts with the homologous antisera. A rather large proportion of rabbits, however, failed to respond by antibody production. Numerous papers have appeared on the inhibition effect of antisera on homologous enzyme antigens (Kirk and Sumner, 1934; Luers and Albrecht, 1926; Smolens and Sevag, 1942). Most of these enzymes have a molecular weight of at least 35,000. *d*-Ribonuclease, however, has about the same molecular weight as lysozyme (about 15,000 to 18,000), and antibody against this enzyme was shown to have some inhibiting effect on the *d*-ribonuclease activity (Smolens and Sevag, 1942). Lysozyme antisera are much more striking in this respect. A similarity exists between lysozyme and *d*-ribonuclease antisera in that a prozone is evident in both systems.

Fleming (1932) suggested that there may be more than one lysozyme. The data presented here would appear to corroborate this observation since almost all

normal rabbit sera have the property of lysing *M. lysodeikticus* and there seems to be no relationship between inherent serum lysozyme activity and the production of lysozyme antibodies. Antiserum prepared against one lysozyme inhibits the lytic activity of only its specific lysozyme, despite the fact that both lysozymes exhibit apparently similar lytic activities. This again demonstrates species-specific differences. Further, it indicates that the chemical groupings, responsible for lytic activity, may differ in the two lysozymes, since the lytic activity of the egg white lysozyme may be completely inhibited, whereas the serum lytic activity is apparently unchanged. There is, of course, always the possibility of a physical blocking of the active lytic groups when antigen-antibody union is effected.

In view of the above it might be desirable either to define individual lysozymes (any material causing the lysis of *M. lysodeikticus*) more fully, or to choose a more appropriate designation for each individual lysozyme. The term "lysozyme" itself, as pointed out originally by Fleming, is ambiguous and certainly not descriptive of any one substance.

Crystalline lysozyme produces partial lysis of *M. lysodeikticus* in dilutions of  $1:15 \times 10^6$  to  $1:20 \times 10^6$ . Serum gives partial lysis in dilutions of about 1:100. If one assumes that the activity per unit weight of each lysozyme is roughly of the same magnitude, then it would appear that the concentration of lysozyme in serum is of the order of 0.01 per cent. It would thus seem that lysozyme is a minor constituent of serum protein, the significance of which is as yet undetermined.

The use of crude egg white lysozyme in man has recently been reported (Ponomareva, 1946a, 1946b). In view of the findings presented here, it might be well to exercise caution in the human experiments since the possibility of sensitization is obvious.

#### SUMMARY

Crystalline lysozyme prepared from egg white is antigenic. It combines with specific rabbit antisera to high titer. Antilysozyme rabbit serum inhibits the activity of lysozyme on *Micrococcus lysodeikticus*.

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# PIGMENT PRODUCTION AND ANTIBIOTIC ACTIVITY IN CULTURES OF *PSEUDOMONAS AERUGINOSA*

G. YOUNG

*Department of Biology, Boston University, Boston, Massachusetts*

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*Pseudomonas aeruginosa* is widely known for two distinctive properties: the production of the blue pigment, pyocyanin, and the ability to lyse or to inhibit the growth of other bacteria. Little attempt has been made to determine the relation between the two, and although pyocyanin and its derivative,  $\alpha$ -oxyphenazine, have been shown to have inhibitory powers (Hettche, 1932; Schoental, 1941), the emphasis has been on antibiotic substances other than the pigments. Hettche has stated, in fact, that no parallelism exists between pigment production and the formation of these other antibiotics, and Gaby (1946) concluded that they were best produced by strains of the organism which formed no pyocyanin at all. These workers, however, paid little attention to the nature of the culture medium and its possible effect on both properties.

Studies of the pigments of *P. aeruginosa* have appeared in the literature with some regularity since Fordos extracted pyocyanin from surgical dressings in 1860. It is interesting that the isolation of the organism itself (Gessard, 1882) was done in an attempt to "verify the parasitic origin of the phenomenon" (i.e., the blue color of pus and dressings) "by M. Pasteur's method of cultures." In addition to pyocyanin, at least four other colored substances are associated with this species: a fluorescent compound, yellow by transmitted light, blue-green by reflected light (Jordan, 1899); pyorubin, a red pigment which is apparently not formed by all strains (Meader *et al.*, 1925);  $\alpha$ -oxyphenazine or hemipyocyanin, a yellow breakdown product of pyocyanin (Wrede and Strack, 1924); and a dark brown substance formed in some old cultures, possibly a derivative of the fluorescent pigment. Of all these, pyocyanin alone has been assigned a useful role in the physiology of the organism. It is easily oxidized and reduced in cultures, and acts in conjunction with the cytochrome system of the cells to increase respiration (Friedheim and Michaelis, 1931; Friedheim, 1931).

The antagonistic power of the organism was shown by Bouchard (1889), when he demonstrated that rabbits injected with anthrax bacilli could be prevented from developing the disease by inoculation with the "blue pus bacillus." Emmerich and Löw (1899), using a culture filtrate to prevent anthrax, believed that the active substance was an enzyme, and named it pyocyanase. The enzyme theory was short lived, as lipoidal, heat-stable extracts were found to be effective (Raubitschek and Russ, 1909), but the name has persisted. Hettche (1934) separated this material into neutral fat, phosphatide, and a mixture of fatty acids, and attributed most of the inhibitory activity to the unsaturated fatty acids. Schoental (1941) obtained, in addition to pyocyanin and  $\alpha$ -oxyphenazine, a pale

yellow oil which showed strong bacteriolytic powers and appeared to be an acid. More recently, Hays and co-workers (1945) have separated a number of active lipoidal fractions, which they have called the "Pyo" compounds.

#### PIGMENT PRODUCTION IN VARIOUS MEDIA

In the present studies, cultures of *P. aeruginosa* from five different sources were used: B, from human feces; S, from a human throat; Ha, from the infected cheek pouch of a hamster; H and T, stock cultures from two different laboratories. Except for slightly more rapid pyocyanin production by strain B, the reactions of all five were identical in the experiments to be described, so no further mention of strain will be made.

TABLE 1  
*Pigment production and acidity in various media*

MEDIUM	pH	PIGMENTS	
		Pyocyanin	Fluorescent
0.5% Glucose	8.12	+	+
1.0% Glucose	6.20	—	—
1.5% Glucose	4.80	—	—
2.0% Glucose	4.18	—	—
0.5% Glycerol	8.50	+	+
1.0% Glycerol	7.57	++	++
1.5% Glycerol	8.10	++	++
2.0% Glycerol	8.26	++	++
Potato + glycerol	8.35	++++	—
Veal infusion	8.40	+	?
Veal infusion + glycerol	8.36	+	?
Blood	8.28	+	?

Nutrient extract broth was used as the base medium with the supplements listed. Cultures were incubated 2 weeks at 37 C.

The first series of experiments was done to determine the effect of various media on pigment production. Nutrient extract broth was used as a base, and the medium was designated by whatever other ingredients were added: 0.5, 1.0, 1.5, and 2.0 per cent glucose; 0.5, 1.0, 1.5, and 2.0 per cent glycerol; potato + 1.0 per cent glycerol; veal infusion; veal infusion + 1 per cent glycerol; and 5 per cent whole blood. The results, including the final pH of each culture, are shown in table 1. Of the five pigments mentioned above, only pyocyanin and the fluorescent pigment are given, because pyorubin was not formed by any of the strains used, and  $\alpha$ -oxyphenazine and the brown substance appear to be formed, not directly by the organism, but indirectly from pyocyanin and the fluorescent pigment, respectively. Pyocyanin was determined by shaking the culture to oxidize the leucobases of the pigments, and extracting with chloroform. Fluorescence was noted on dilution of a small amount of the culture after chloroform extraction.

It appears that pigment production is associated with alkalinity, and if enough

glucose fermentation takes place so that the reaction remains acid no colored substances are formed. Cultures containing small amounts of glucose—up to 1 per cent—show an acid reaction during the first week of incubation, and then gradually become alkaline, with some pigment formation. That the pigments are not already present in some colorless form is shown by the fact that when NaOH is added to an acid culture and the pH brought to 8.0, there is no immediate evidence of pigment, but in a few days both pyocyanin and the fluorescent material begin to appear. The slight amounts of pigments formed in veal infusion and blood broths, which give distinctly basic cultures, indicate that alkalinity is not the only factor involved, and that enriched media of this type inhibit rather than encourage pigment production, even in the presence of glycerol. The addition of glycerol alone favors the production of both pigments, and potato further increases pyocyanin formation, but appears to inhibit the fluorescent material. (The reaction of all these cultures is remarkably constant after the first 2 weeks; 10-week cultures, either acid or basic, show the same pH as at 2 weeks, although such properties as color and viscosity may have changed considerably. In no case do the organisms die out.)

#### ANTIBIOTIC ACTIVITY IN VARIOUS MEDIA

Three of these media were chosen for further work: 1.5 per cent glucose, in which no pigments were produced; potato + 1 per cent glycerol, in which pyocyanin was formed; and 1 per cent glycerol, which yielded both pyocyanin and the fluorescent substance. Samples were taken from cultures after 1, 4, and 10 weeks of incubation; each was sterilized by boiling for 1 minute and 3 ml were added to a tube of melted nutrient agar. Plates were poured and streaked with three test organisms, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium smegmatis*. After 24 hours the growth of *S. aureus* and *E. coli* on the test plates was compared with that on a control plate of nutrient agar. Because *M. smegmatis* grows more slowly, both test and control plates were replaced in the incubator for another 24 hours before the growth of that organism was recorded. The results are given in table 2. It is clear that the 1.5 per cent glucose culture, producing no pigments, is also lacking in antibiotic activity, whereas the other two media are effective in both respects. The diminishing inhibition of *E. coli* by older cultures will be referred to later.

#### PROPERTIES OF CULTURE FRACTIONS

In order to analyze further the relation between pigment formation and antibiotic potency, cultures in the three media were allowed to incubate for 3 weeks and then fractionated. The 100-ml broth culture was shaken in order to oxidize the leucobases of both pyocyanin and the fluorescent pigment. It was then extracted with chloroform (4 successive 20-ml portions) to remove pyocyanin and any small amounts of  $\alpha$ -oxyphenazine present. Because pyocyanin breaks down rapidly into the latter substance in chloroform solution, this extract was immediately treated with very dilute hydrochloric acid (three 10-ml portions), pyocya-

nin appearing in its red, acid form in the water layer. The pH of the aqueous fraction was adjusted to 7.0, and the concentration read colorimetrically. The chloroform layer was evaporated under suction, and the residue of  $\alpha$ -oxyphenazine was taken up in ether (10 ml) and also read colorimetrically. The culture was then acidified to pH 4.0 and centrifuged to remove the resulting flocculent white precipitate and the cells. (In fluorescent cultures the acid supernatant was a bright orange color.) Both precipitate and supernatant were extracted with ether to remove fatty acids; the extracts were combined and reduced in volume to 20 ml. After extraction the supernatant was neutralized. The fluorescent pigment remained in this residue and regained its blue-green color on neutralization.

TABLE 2  
*Relation of pigment production to antibiosis*

MEDIUM	PIGMENTS		INHIBITORY EFFECT*		
	Pyocyanin	Fluorescent	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smogmatis</i>
1.5% Glucose					
1 week	—	—	+++	+++	+++
4 weeks	—	—	+++	+++	+++
10 weeks	—	—	+++	+++	+++
Potato + glycerol					
1 week	+	—	—	—	—
4 weeks	+	—	—	—	—
10 weeks	+	—	—	++	—
1% Glycerol					
1 week	+	+	—	—	—
4 weeks	+	+	—	+	—
10 weeks	+	+	—	+++	—

\* Three ml of heated culture in 10 ml of agar. Growth and inhibition of test organisms are indicated by + and — signs, respectively.

Since the solid portion of the culture was found to be inactive after extraction, the four fractions tested were the water-soluble and ether-soluble fractions of the chloroform extract, the combined ether extracts from the acidified culture after chloroform extraction, and the cell-free culture residue. The final concentration of each fraction was chosen to give an end point in tests of inhibitory action, with amounts which could be added to 10 ml of agar. This means, of course, that although the potencies of any one fraction may be compared from one test to another, the different fractions are not comparable with one another.

Pigments and antibiotic activities for these fractions obtained from cultures in the three types of media are given in table 3. Four important facts emerge: the total absence of antibiotic effect in cultures in which no pigment is produced; the association between pyocyanin and the inhibition of *E. coli*; the removal of some



TABLE 3  
*Pigment production and antibiotic activity of culture fractions*

MEDIUM	CHLOROFORM EXTRACT				ETHER EXTRACT		RESIDUE NEUTRALIZED	
	Water-soluble fraction (Pyocyanin)		Ether-soluble fraction ( $\alpha$ -oxyphenazine)		From acidified culture (fatty acids)		Pigment	Inhibition
	Pigment	Inhibition	Pigment	Inhibition	Pigment	Inhibition		
1.5% Glucose	—	—	—	—	—	—	—	—
Potato + glycerol	++++ Blue	<i>E. coli</i> <i>S. aureus</i> <i>M. smegmatis</i>	+	<i>S. aureus</i> <i>M. smegmatis</i>	Very slight yellow	<i>S. aureus</i> <i>M. smegmatis</i>	—	—
1% Glycerol	++ Blue	<i>E. coli</i> <i>S. aureus</i> <i>M. smegmatis</i>	+	<i>S. aureus</i> <i>M. smegmatis</i>	Very slight yellow	<i>S. aureus</i> <i>M. smegmatis</i>	+++ Fluorescent	<i>S. aureus</i> <i>M. smegmatis</i>

antibiotic material from acidified cultures by ether (matter which is not extracted from alkaline cultures by chloroform), thus confirming the fatty acid hypothesis; and the presence of some water-soluble inhibitory substance, not hitherto described, in residues containing the fluorescent pigment.

This last observation is especially significant in view of the statements

TABLE 4  
Effect of time on the antibiotic activity of culture fractions

WEEK	AMT.	WHOLE CULTURE			PYOCYANIN			OXYPHENAZINE			ETHER EXTRACT			FLUORESCENT RESIDUE		
		<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>
1	ml.															
	4	-	-	-	-	-	-	-	+++	-	-	+++	-	-	+++	-
	3	-	-	-	-	-	-	-	+++	-	-	+++	-	-	+++	-
	2	-	++	-	-	+	-	-	+++	-	+	+++	-	+	+++	+
	1	-	+++	-	-	+	-	++	+++	-	++	+++	+	+	+++	+
2	0.5	+	+++	-	+	+	-	+++	+++	+	+++	+++	++	++	+++	++
	4	-	-	-	-	-	-	-	+++	-	-	+++	-	-	+++	-
	3	-	-	-	-	-	-	-	+++	-	-	+++	-	-	+++	-
	2	-	-	-	-	-	-	++	+++	+	+	+++	-	-	+++	-
	1	-	+	-	-	+	-	++	+++	+	++	+++	+	-	+++	-
3	0.5	+	+++	-	+	+	-	+++	+++	++	++	+++	+	+	+++	-
	4	-	-	-	-	-	-	-	+++	-	-	+++	-	-	+++	-
	3	-	-	-	-	-	-	-	+++	-	-	+++	-	+	+++	-
	2	-	-	-	-	-	-	-	+++	-	+	+++	+	+	+++	-
	1	-	-	-	-	-	-	++	+++	+	++	+++	+	++	+++	+
4	0.5	+	++	-	-	+	-	++	+++	+	++	+++	+	++	+++	+
	4	-	-	-	-	-	-	-	+++	-	-	+++	-	+	+++	-
	3	-	+	-	-	-	-	-	+++	-	-	+++	-	+	+++	+
	2	-	++	-	-	+	-	+	+++	-	+	+++	-	++	+++	++
	1	-	+++	-	-	+	-	++	+++	+	+	+++	-	+++	+++	+++
6	0.5	++	+++	+	+	++	+	++	+++	++	++	+++	+	+++	+++	+++
	4	-	+	-	-	+	-	-	+++	-	-	+++	-	+	+++	+
	3	-	++	-	-	+	-	-	+++	-	-	+++	-	+	+++	+
	2	-	+++	-	-	++	-	+	+++	-	-	+++	-	++	+++	+
	1	-	+++	-	+	+++	+	++	+++	++	+	+++	+	++	+++	++
10	0.5	+	+++	+	++	+++	++	++	+++	+++	++	+++	+	+++	+++	++
	4	-	+++	-	-	+	-	-	+++	-	-	+++	-	+	+++	-
	3	-	+++	-	-	++	-	-	+++	-	-	+++	-	+	+++	+
	2	-	+++	-	-	+++	-	+	+++	-	-	+++	-	++	+++	++
	1	-	+++	+	+	+++	+	++	+++	++	-	+++	-	++	+++	++
10	0.5	+	+++	+	+++	+++	+	+++	+++	+++	+	+++	+	+++	+++	++

(Hettche, 1932; Schoental, 1941) that all antibiotic material produced by this organism is removed by fat solvents. The substance is heat-stable, and it seems likely that it is identical with the inhibitor produced by other fluorescent bacteria. Antibiotic activity in this group of organisms has been described by several authors. Garré (1887) used *Pseudomonas putida* to inhibit *S. aureus* and other organisms, and Frost (1904) found both *Pseudomonas fluorescens* and *Pseudomonas putida* bactericidal for *Eberthella typhosa*. Lewis (1929) showed

that *P. fluorescens* was effective against sporeforming soil bacteria and micrococci, but not against *E. coli* or *Serratia marescens*.

#### THE EFFECT OF TIME ON PRODUCTION OF PIGMENTS AND ANTIBIOTIC ACTIVITY

To determine the effect of time on the production of these fractions, cultures in glycerol broth were analyzed after 1, 2, 3, 4, and 10 weeks of incubation. The results, shown in table 4, indicate that several changes occur as the time of incubation is increased. The inhibition of *E. coli* by whole cultures and by pyocyanin decreases rapidly after the third week. A comparison of the effect on

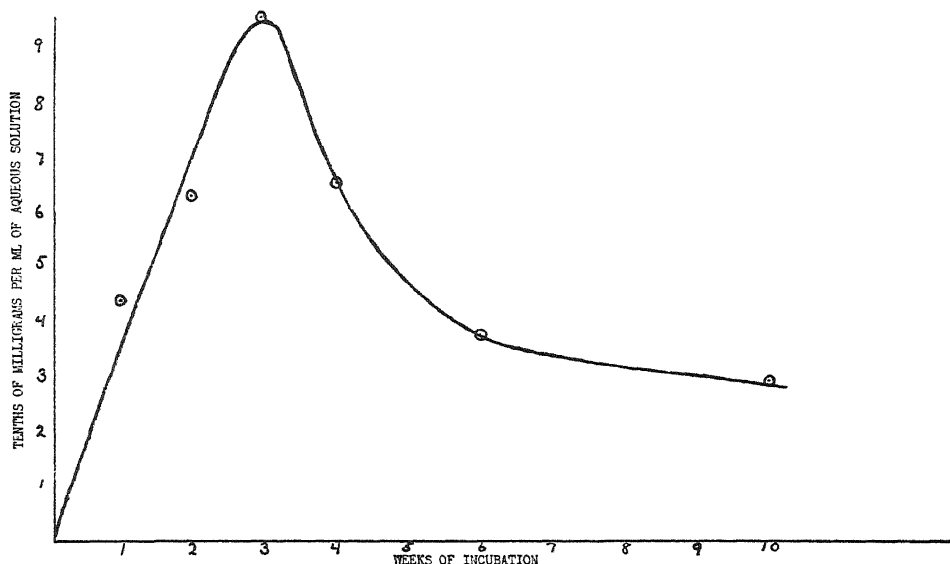


FIG. 1. RELATION BETWEEN PYOCYANIN CONTENT AND AGE OF CULTURE  
(ONE PER CENT GLYCEROL BROTH MEDIUM)

this organism with the actual concentration of pyocyanin (figure 1) shows a direct proportion. The actual amount of  $\alpha$ -oxyphenazine extracted is small and proportional to the amount of pyocyanin. The ether extract, on the other hand, shows a growing effectiveness in older cultures. An increase in this factor at the time when pyocyanin is decreasing would explain the continued inhibition of *S. aureus* and *M. smegmatis* by whole cultures. The activity of the fluorescent residue is greatest in young cultures, diminishing after the second week.

#### SUMMARY

*Pseudomonas aeruginosa* produces no pigments in culture media containing sufficient glucose (over 1 per cent) to establish and maintain an acid reaction. Pyocyanin is profusely formed in potato glycerol broth, and in glycerol broth both pyocyanin and a fluorescent pigment are produced. Enrichment of the medium

with veal infusion or blood inhibits pyocyanin formation even in the presence of glycerol.

Acid cultures of *P. aeruginosa*, which produce no pigments, form no antibiotic substances of any kind.

Pyocyanin inhibits *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*. The last two organisms are also inhibited by  $\alpha$ -oxyphenazine, ether extracts of acidified cultures, and by chloroform-extracted and ether-extracted culture residues which contain fluorescent pigment. This water-soluble, heat-stable antibiotic which accompanies fluorescence may be the same as that responsible for inhibitory action of other species of fluorescent bacteria.

Pyocyanin in chloroform solution breaks down rapidly into  $\alpha$ -oxyphenazine. When it is removed into water solution immediately after extraction, very small amounts of the latter substance are found.  $\alpha$ -Oxyphenazine should therefore not be considered as contributing to the natural antibiotic activity of *P. aeruginosa*.

In glycerol broth, pyocyanin production rises to a peak at 3 weeks of incubation and then drops off. The fatty acid fraction becomes more effective in older cultures, whereas the inhibitory material of fluorescent residues is most abundant at 2 weeks.

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# THE MODE OF ACTION OF NITROFURAN COMPOUNDS

## II. APPLICATION OF PHYSICOCHEMICAL METHODS TO THE STUDY OF ACTION AGAINST STAPHYLOCOCCUS AUREUS

D. L. CRAMER<sup>1</sup>

*Research Division of the Eaton Laboratories, Norwich, New York*

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The first report of this series called attention to the fact that, of a group of varied nitrofurans, the 2-(5-nitro)-furaldehyde semicarbazone, named "furacin," was distinctly different from the others in its mode of antibacterial activity when tested with a coagulase-positive strain of *Staphylococcus aureus* (Cramer and Dodd, 1945). We have further examined the effect of furacin upon the oxidation-reduction potential of growing cultures of a coagulase-positive staphylococcus, and also the effect of eventual growth upon the concentration of furacin. We have found that a poisoning of  $E_h$  does occur, and that subsequently if growth takes place the chemical compound is reduced, undoubtedly at the 5-nitro group. We wish to present data related to these events, and to discuss the implications with respect to the vital processes of the bacteria.

These data have been obtained by the application of purely physicochemical methods, and at least in the instance of the polarographic method of analysis of bacterial cultures, represent an unusual and simple approach to the determination of a single constituent in the complex mixture that results from bacterial growth, without the necessity of detailed separation procedures.

### EXPERIMENTAL

The changes of potential occurring in growing cultures were measured by means of a simple potentiometer, a Leeds and Northrup type  $K_1$  (Hewitt, 1936). Electrodes were made by sealing 22-gauge platinum wire into 2-mm soft glass tubing; mercury was used to connect the Pt electrode to the potentiometer circuit. The culture vessels were 25-mm pyrex test tubes. The electrode and an inverted U-tube, the latter to serve as an electrical bridge, were rolled into a cotton plug and sterilized by autoclaving, following which the bridge was filled aseptically with a sterile, saturated KCl solution containing 3 per cent agar. For experimental observations exactly 25 ml of the medium to be examined were placed in a sterile culture vessel and inoculated with 50,000 to 100,000 organisms per ml, and the electrode- and bridge-containing plug was inserted. The vessel was then placed in a water bath at 37 C, the outer tip of the KCl-agar bridge being placed in a reservoir of saturated KCl solution. Into this reservoir also was placed the side arm of a saturated calomel electrode, and the cell thus formed was connected to the potentiometer. It was determined that the calomel half-cell under the

<sup>1</sup>The author wishes to express appreciation for the generous counsel and encouragement of Dr. M. C. Dodd throughout the course of this investigation.

conditions used would produce an emf of +250 millivolts. Thus, experimental readings in millivolts, obtained during growth, were referred to the hydrogen electrode, i.e., converted to  $E_h$ , by adding +250 to the observed reading.

The furacin present in culture media may be quantitatively estimated by the polarographic technique (Kolthoff, 1941). The 5-nitro group of the furacin molecule is reducible at the dropping mercury electrode. The half-wave potential observed for aqueous solutions buffered at pH 7.0 is -0.4 volt, against a saturated calomel external anode, and the current-voltage curve for the compound is flat in the range -0.6 to -1.0 volt. If, therefore, one makes a polarogram of a culture medium, e.g., broth, both with and without a known quantity of furacin present, the difference in diffusion current at -0.8 volt, or  $\Delta i_{d^{0.8}}$ , should be proportional to the amount of furacin present. The technique is particularly suitable for our purposes; it is sufficiently sensitive, accurate, and is relatively unaffected by turbidity, particulate material, or compounds present in addition to furacin.

For any particular sample of culture material the procedure of analysis consists in heating the sample to 56 C for a period of 35 minutes to 1 hour, to kill any organisms present and to stop enzyme reactions, then making a polarogram in the range 0 to -1.0 volt. The sample must be deaerated by bubbling pure nitrogen through the medium for 15 minutes. The difference in the sample and suitable controls permits one to observe the concentration of the nitro compound. We have expressed the concentration of furacin remaining in solutions

as the percentage of residual furacin, or the ratio: 
$$\frac{\Delta i_{d^{0.8}} \text{ final}}{\Delta i_{d^{0.8}} \text{ initial}}$$

This seemed advisable in comparative data such as we present, since it obviates correction of diffusion current values which may change slightly during the course of several experiments because of changes in temperature, fluctuations in drop rate, and similar variables. An Aminco polarometric analyzer was used in these determinations.

The medium used in this investigation was Difco brain heart infusion broth. The organism was a recently isolated, coagulase-positive strain of *S. aureus*. Inoculations were made from 24-hour broth cultures of the organism, using 50,000 to 100,000 organisms per ml of the medium to be examined.

#### RESULTS

*$E_h$  measurements.* The data from a representative experiment showing the change of  $E_h$  during growth in the control, in two samples containing different bacteriostatic concentrations, and in a sample containing bactericidal concentration of furacin are presented in table 1. The concomitant change of pH in the control was found to be 0.4 to 0.5 units, e.g., from 7.20 to 6.75. It would be desirable, in similar future experiments to measure pH simultaneously with  $E_h$ . Such refinement, with subsequent corrections, has not been done in this work. An idealized diagram for comparison of the concentration effect appears as figure 1.



From consideration of figure 1 it is evident that the principal effect of increasing the concentration of furacin is to prolong the time before the initial  $E_h$  will drop.

TABLE 1

*Change of  $E_h$  during growth of *S. aureus* in broth containing furacin*

HOURS AFTER INOC.	CONTROL		1/200,000		1/100,000		1/50,000	
	$E_h$ , mv	Growth	$E_h$ , mv	Growth	$E_h$ , mv	Growth	$E_h$ , mv	Growth
0	+157		+170		+166		+100	
1	164		190		176		96	
2	164		202		175		98	
3	156		190		179		102	
4	111	+	168		176		109	
5	107		168		170		103	
6	7	++	168	trace	167		102	
7	20		111		163		95	
8	10	+++	88	++	147		91	
9	15		6		125		77	
10	10		5		117			
11	+2		30		108			
12	-2	++++	+20			trace		
15	-30		-10	++++	45	++	80	
18	-63		-40		+7		80	
24	-88		-65		-28	++++	85	
48	-92		-110		-95		80	
96	-85		-105		-100		70	-

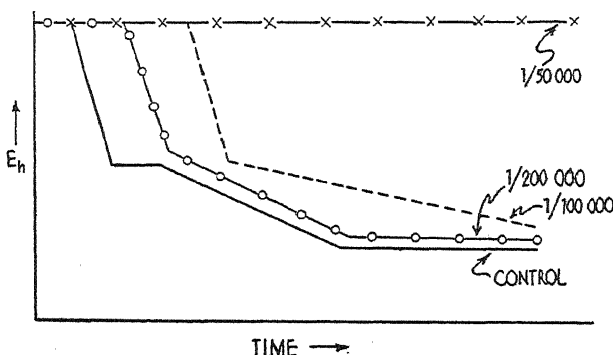


FIG. 1. CONCENTRATION EFFECT OF 2-(5-NITRO)-FURALDEHYDE SEMICARBAZONE UPON THE  $E_h$  OF GROWING *STAPHYLOCOCCUS AUREUS*

- control.  
 -X-X- 2-(5-nitro)-furaldehyde semicarbazone 1:50,000.  
 — 2-(5-nitro)-furaldehyde semicarbazone 1:100,000.  
 -O-O- 2-(5-nitro)-furaldehyde semicarbazone 1:200,000.

With a bactericidal concentration, this initial value does not significantly change. With simply bacteriostatic concentrations the initial level is maintained for a time period in excess of that in the control.

Change in concentration of furacin during growth. Table 2 and figure 2 show current-voltage data for broth—broth in which *S. aureus* has grown fully and broth containing furacin at a concentration of 1:100,000.

TABLE 2  
Current-voltage relations for furacin in broth

EMF., -VOLT	BROTH	BROTH AFTER FULL <i>S. aureus</i> GROWTH	FURACIN 1/100,000 IN BROTH
0.0	-3.3	-3.1	-3.3
0.2	-1.5	-1.5	-1.6
0.3	-1.4	-1.4	-1.4
0.4	-1.3	-1.35	-1.3
0.5	-1.3	-1.3	-0.7
0.6	-1.3	-1.3	-0.6
0.8	-1.05	-1.05	-0.35
1.0	-0.75	-0.65	0.0

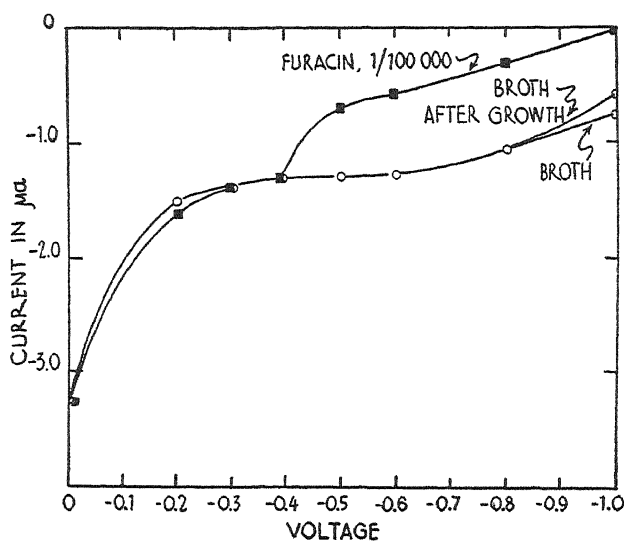


FIG. 2. POLAROGRAMS FOR BROTH AND 2-(5-NITRO)-FURALDEHYDE SEMICARBAZONE IN BROTH

—○—○— broth.

—■—■— 2-(5-nitro)-furaldehyde semicarbazone in broth, concentration, 1:100,000.

The effect of added furacin is obvious. The average difference in the diffusion current at  $-0.8$  volts due to the presence of 1:100,000 furacin is  $0.7 \mu a$ , i.e.,  $\Delta i_d^{0.8} = 0.7 \mu a = 1:100,000$ . Similarly, we have determined that for a concentration of 1:50,000  $\Delta i_d^{0.8} = 1.4 \mu a$ , and for 1:25,000  $\Delta i_d^{0.8} = 2.75 \mu a$ . Thus, for this range of concentration, the amount of furacin is a linear function of  $\Delta i_d^{0.8}$ . The average value for the dilution of furacin producing  $\Delta i_d^{0.8} = 1 \mu a$

under these conditions is 1:70,000. This is the quantitative analytical basis for estimating furacin in bacterial culture media.

Table 3 shows representative data for the amount of furacin remaining in a culture of *S. aureus* growing in the presence of 1:100,000 furacin; visible growth indication is also included.

TABLE 3  
*Changes in concentration of furacin during growth*  
(Initial concentration furacin = 1:100,000)

HOURS AFTER INOC.	$\Delta \text{id}_{50}$ , $\mu\text{g}$	RESIDUAL FURACIN	VISIBLE GROWTH
		%	
0	0.7	100	--
2	0.7	100	--
4	0.7	100	--
6	0.65	93	--
8	0.55	79	--
10	0.50	71	--
12	0.45	65	trace
15	0.25	36	++
18	0.15	21	++++
24	0.10	14	++++

#### DISCUSSION

It has long been known that in broth *S. aureus* has a lag period of 1 to 2 hours, followed by a rapid growth that is complete in 8 to 9 hours. The effect of furacin in a concentration of 1:100,000 is to prolong the lag period, in this case the prolongation being at least 6 to 7 hours. We have now demonstrated that during this prolonged lag the  $E_h$  of the culture remains poised. Moreover, during subsequent growth, furacin disappears.

The poisoning of potential, followed by what seems to be a normal drop in  $E_h$ , confirms our previous conclusion (Cramer and Dodd, 1945) that the only effect observable in population curves of this particular drug-organism combination occurs during the lag phase. It may well be that the poisoning of the  $E_h$  at a point unfavorable to growth is the primary cause for bacteriostasis, in line with the suggestion of Dubos (1929) concerning crystal violet.

It is also apparent that an induction period is necessary before the reduction of the 5-nitro group of the furacin molecule occurs (table 3). With furacin at a concentration of 1:100,000 the organism under these conditions has a lag period of 8 to 9 hours. Reduction, however, is initiated 5 to 7 hours after inoculation, and at 9 hours is approximately 25 per cent complete. This suggests that reduction is initiated shortly before the lag period is ended. This would appear to indicate that at least the initiation of reduction may be necessary before growth can take place, and may signify that furacin must be reduced below a critical level before growth starts.

It is only with difficulty that the time relations in experiments designed to show the change in  $E_h$  with growth, or the rate of reduction of furacin, may be duplicated within a narrow range. Successive cultures of the same organism, and also successive lots of the same nutrient material, show a considerable effect in varying the hour at which an expected change occurs. However, the over-all trend of these changes with time remained the same in successive repetitions of our experiments.

We propose then this sequence of events for a culture growing in a bacteriostatic concentration (1:100,000) of furacin: (a) growth is delayed, as manifested by a prolonged lag and a poised  $E_h$ ; (b) reduction of furacin begins; and (c) growth is initiated and proceeds at a normal rate. The latter event is accompanied by a drop in  $E_h$ , and the reduction of furacin in an apparent zero order reaction.

Hinshelwood (1944) has recently reviewed various factors influencing events of the bacterial growth cycle. He has pointed out the necessity, early in the lag phase, of a regeneration of inactivated enzymes and a fresh accumulation of labile or diffusible intermediates responsible for the extension of enzyme patterns prerequisite to logarithmic growth. Certain correlations of the phenomena we observe with these vital events can be made. The induction period before the reduction of furacin is probably explained at least in part by the reactivation of an enzyme. However, it is probable that the effective concentration of reactivated enzyme is in the beginning so small that the reduction in the amount of furacin, although it may start soon after the introduction of furacin into the system, is so slow that by our present methods differences in furacin concentration are not measured. When the enzyme is fully reactivated, as for example, 5 hours after inoculation of broth containing 1:100,000 furacin (table 3), which is roughly midway through the extended lag period, then the reduction is proceeding at its maximum velocity, having probably changed, in our opinion, from a first order to a zero order reaction. Under these conditions the change in the concentration of furacin becomes measurable, as the data show. The diversion of at least a part of this enzyme activity from normal metabolic processes into the reduction of furacin must result in an interference with the production by one enzyme of a sequence of an intermediate utilized by a later member of the sequence. This would be an uncomplicated explanation. This interference is manifested as a temporary delay in the completion of the necessary extension of enzyme patterns that must occur before growth becomes logarithmic. When either the velocity of such an extension becomes great enough that the reaction rate of furacin reduction is no longer critical, or when the concentration of furacin itself is lowered beyond a critical level, then growth proceeds simultaneously with a continued reduction, and appears unaffected by that reduction. It must be pointed out again that, under such conditions as we have defined, the effective events in the antibacterial action of furacin have all occurred by the time that the lag phase has ended.

Thus, as previously suggested (Cramer and Dodd, 1945), furacin does indeed

interfere with a normal metabolic process of the organism, and does so by virtue of its capabilities of being reduced. These facts emphasize the importance of a better knowledge of the reducing system present in the organism, which for convenience may be called "furacin reductase," since susceptibility may be intimately linked to this factor. Such information we hope to obtain.

We wish to emphasize the utility of physiochemical methods in this study, particularly the polarographic method. The only technique previously available for quantitative determination of nitrofurans utilized spectral absorption in the near ultraviolet. This proved quite unsatisfactory for use in turbid, protein-containing culture liquors. On the other hand, the polarographic technique, when applicable to the compound in question, does not suffer unduly from such interfering influences. Moreover, in practice it proved remarkably simple in regard to operations necessary to prepare a viable culture for analysis.

#### SUMMARY

Examination of the effect of 2-(5-nitro)-furaldehyde ("furacin") upon changes in the oxidation-reduction potential of a growing culture of *Staphylococcus aureus* has revealed that a poisoning occurs.

By means of a polarographic method of analysis for furacin in bacterial culture liquors it has been demonstrated that it is destroyed by bacteria. A mechanism for such destruction is suggested.

The implications of the time relationships between such changes upon the bacterial growth process were discussed.

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# CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

## II. CHANGES IN REACTIONS OF STAPHYLOCOCCUS AUREUS TO VITAL DYES<sup>1</sup>

ROBERTSON PRATT AND JEAN DUFRENOY<sup>2</sup>

*University of California College of Pharmacy, The Medical Center,  
San Francisco 22, California*

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In a previous paper we described the gross appearance of penicillin assay plates after chemical treatments which reveal sharp boundaries around the zones of inhibition (Dufrenoy and Pratt, 1947). Most of the tests that were described are effective following exposure of the test organisms to penicillin for periods as short as 2 to 3 hours, i.e., before zones of inhibition are discernible on untreated plates. Several of the tests may find application as rapid cylinder plate assay methods (Pratt and Dufrenoy, 1947). The sharp delineation of the inhibition zones was interpreted as an expression of a threshold effect; positive reactions for —SH groups and for  $\text{OH}=\text{C}-\text{C}=\text{OH}$  groups were obtained outside the zones of inhibition, but not inside. The evidence suggested an increase of rH within the zones and that this increase was correlated with inhibition of dehydrogenase systems. Since there is strong evidence in the literature that dehydrogenase activity depends on the structural integrity of the microorganism (Guggenheim, 1945), it seemed of interest to study the changes in response to various reagents in different parts of the cells of test organisms following exposure to penicillin. In this paper cytochemical structure will be interpreted from microscopical study of living cells of *Staphylococcus aureus* treated with vital stains, and from observations made after treatment of the organisms with various other chemical reagents. Our experimental data confirmed the data available in the literature to the effect that the part within the living cells of *Staphylococcus aureus* which responds most evidently to various reagents represents the vacuolar materials.

The accumulation of vital dyes in the vacuole may be correlated with the presence and nature of phenolic compounds within the vacuolar solution in a twofold manner: first, because phenolic compounds, and in general dienol compounds, play a fundamental role in the respiratory systems providing the energy for active absorption; secondly, because some actual linkage may occur. Historically it may be noted that as early as 1900 Nakanishi recognized that living and dead staphylococci respond differently to staining with methylene blue.

Imsenecki (1946) has pointed out that "many authors, Piekarski, Peshov, Robmorv,—found in long cells of *Proteus vulgaris*, chromophilic granules which they regard as nuclei . . . the structures mistaken for nuclei are polar granules,

<sup>1</sup> The execution of the work reported in this paper was made possible by a generous research grant from the Cutter Laboratories, Berkeley, California.

<sup>2</sup> With the Laboratory Assistance of Toinie Juntunen.

an excellent picture of which was given by Migula in 1897." Migula indeed clearly understood the significance, if not the chemical nature, of polar granules in bacteria, and their relation to vacuoles, which he discussed in his *System der Bakterien*. These vacuolar granules are now recognized as involving Feulgen-negative material in contrast with the nuclear part of the cell which is Feulgen-positive. In addition to the Feulgen-negative bodies, the vacuolar material contains phenolic compounds.

Among the vital stains used, some accumulate almost exclusively in the vacuolar solution, others may be partly adsorbed by other cell constituents. Accumulation of a dye in the vacuolar solution is influenced by the quantity and nature of the phenolic compounds that are present.

#### MATERIALS AND METHODS

Assay plates were prepared according to the standard method prescribed by the FDA. Some were incubated according to the standard 16-hour method. Others were seeded, preincubated for 3 hours, cylindered, and then subjected to a second incubation period of 3 hours with penicillin, according to the modified cylinder plate method involving physical development (Goyan, Dufrenoy, Strait, and Pratt, 1947). The test organism used was *Staphylococcus aureus* NRRL strain 313 (same as FDA strain 209P).

For cytological examination, organisms were removed with a platinum loop from different parts of test plates and were suspended in a drop of a solution of a vital dye or other reagent without previous killing and fixing. Cells collected from the uninhibited background outside the zones of inhibition (normal cells) were compared with those taken from different locations within zones surrounding cylinders containing solutions with concentrations of penicillin ranging from 0.5 to 8 Oxford units per ml.

These cells were compared with cells removed from similar locations on plates which subsequent to incubation with penicillin were flooded with the several reagents. The various regions of the treated plates were also examined directly under the oil immersion to study the pattern of response to the several reagents within cells retaining their original position in relation to the gradient of penicillin diffusing from the cups. These studies *in situ* confirmed in every case the cytological observations made on test organisms transferred from homologous sites on the plates to glass slides before addition of the reagent. The vital dyes used most extensively were neutral red, methylene blue, and methyl green.

#### EXPERIMENTAL RESULTS

Fisher (1946) documented his "Study on the mechanism of action of penicillin as shown by its effect on bacterial morphology" by means of excellent photographs, some of which illustrate the results of staining with a dilute solution of carbol fuchsin—a technique which apparently was initiated by Nakanishi (1900) and which was used by Stoughton in his attempts to demonstrate the presence of a nucleus in *Bacterium malvacearum* (1929, 1932). Fisher's photographs show



that each "control" cell contains a stained body. We interpret this stained body as representing a drop of vacuolar solution, such as has been shown to respond to vital staining as well as to staining with the carbol fuchsin technique (Dufrenoy, 1931), and such as we have found in our present work with penicillin.

Our own researches with vital staining showed that when cells of the test organisms are exposed to penicillin, the vacuolar solution tends to be displaced from its central position toward the margin of the cell, and generally toward one pole. Consequently, dyes which normal staphylococcus cells would concentrate in the central vacuole tend to accumulate more and more toward the periphery, and preferentially toward one pole, in cells under the influence of penicillin. Eventually the swollen cells may assume the appearance of a stained shell (figure 1).

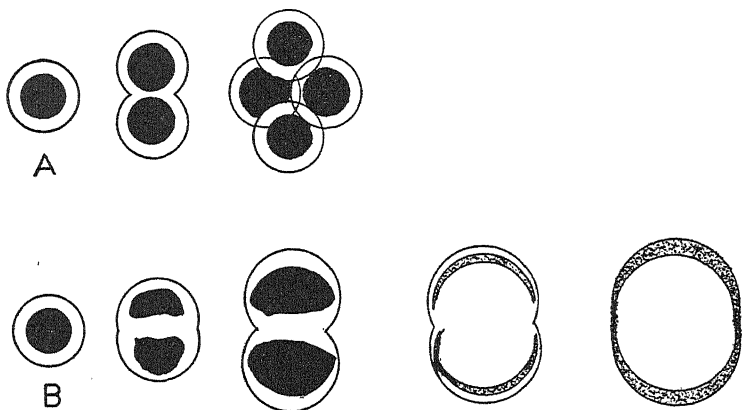


FIG. 1. DIAGRAMMATIC REPRESENTATION OF *STAPHYLOCOCCUS AUREUS* STAINED VITALLY WITH NEUTRAL RED

A. Phases of cell division in normal *Staphylococcus aureus*. Vacuolar material which selectively stains red is shown as solid black.

B. Phases of unsuccessful attempt toward a first division of *Staphylococcus aureus* under the influence of a bacteriostatic concentration of penicillin. Successive diagrams from left to right show that the cell swells; material staining with neutral red diffuses from the vacuole to the periphery; the cell fails to divide and eventually swells into an empty shell, which exhibits diffuse bipolar peripheral staining (stippled area).

It is recognized that penicillin acts on *S. aureus* as it is actively dividing and may allow a first division to occur. Figure 1A represents diagrammatically the behavior of a normal, living cell of *S. aureus* exposed to a solution of a vital dye. It may be seen that the dye accumulates in the vacuole and that cell division proceeds without hindrance. Figure 1B is a similar representation of the behavior of cells subjected to a vital dye following exposure to a bacteriostatic concentration of penicillin. The initial stages are about normal, but, instead of each of the two resulting vacuoles being distributed to daughter cells, the vacuolar material (staining with basic dyes) tends to move to the periphery of the swelling parent cell, which often fails to complete division.

It will be recalled here that the vacuolar accumulation of vital dyes against a concentration gradient in normally functioning aerobic organisms is a physiologi-

cal process entailing expenditure of energy that is derived from respiration; as respiration becomes unbalanced, the vacuolar solution not only becomes unable to absorb materials from the external environment, but even becomes unable to retain the solutes it already contains. Thus its solutes are free to seep out. This condition, which is one of the early symptoms of the effect of penicillin on susceptible cells, occurs concomitantly with the disorganization of the cellular nucleoproteins and the liberation of lipids and fatty acids.

The disorganization of cellular constituents is notably revealed by the "lipophaneresis," defined by Lison (1936) as the unmasking of the reactive groups of the fatty components from the liponucleic complex. Hurst (1945) pertinently remarked that "lethality usually involves an irreversible increase in phenoloxidase activity, produced by the displacement of protective lipoids from the tissue receptors."

Data previously reported from assay plates (Dufrenoy and Pratt, 1947) demonstrated that bacteriolytic or bacteriostatic effects of penicillin are correlated with a relative increase of phenoloxidase activity that occurs concomitantly with an inactivation of dehydrogenase systems. This inactivation may be cytochemically visualized as resulting from displacement of the protective lipoids from the lipoproteins in the dehydrogenase systems.

In the present investigation it has been shown experimentally by evidence from standard 16-hour assay plates treated with appropriate reagents (Nile blue, copper acetate, or fat-soluble dyes) that lipids are displaced from cells undergoing lysis in the inhibition zones surrounding cylinders containing solutions of penicillin ranging in concentration from 0.25 to 8 units per ml, and that the liberated lipids are hydrolyzed into fatty acids. It is well known that basic dyes which are able to combine with fatty acids form soaps which have the color of the salt of the dye. The sharpest response was obtained with Nile blue, which Knaysi (1941) recommended for the discrimination of neutral fats from fatty acids.

When standard 16-hour assay plates are flooded for 1 minute with a saturated aqueous solution of Nile blue, and then are rinsed with distilled water, normal colonies (uninhibited areas of the plates) stain a deep blue and stand out boldly from the agar substrate. Where lysis has occurred, within the zones of inhibition, a purple color develops. This area of lysis is surrounded by a clear blue ring corresponding to the region of enhanced growth just beyond the threshold of bacteriostatic concentration of penicillin.

A convergent line of evidence that fatty acids are liberated at the site of bacteriolysis is furnished by flooding 16-hour assay plates with a saturated aqueous solution of copper acetate and further incubating them for 6 hours at 37 C. When this is done, a thick, opaque layer of bluish copper salts of fatty acids develops covering entirely the areas of the inhibition zones. The areas of uninhibited growth do not appreciably react with the reagent. The reactive and nonreactive regions are sharply delineated.

Further support for the conclusions drawn from the results obtained with Nile blue and copper acetate was provided by experiments with FD and C yellow

no. 3,<sup>3</sup> a fat-soluble dye which stains neutral fat a bright yellow and imparts a deep orange color to fatty acids. Plates flooded with a saturated solution of the dye in methylal develop a bright orange color where lysis has occurred. Each zone of lysis is surrounded by a bright yellow ring delineating the region of enhanced growth.

#### DISCUSSION AND CONCLUSIONS

In previous papers (*loc. cit.*) we demonstrated that suitable reagents applied to penicillin assay plates under appropriate conditions sharply delineate the general background of uninhibited growth from the zones of inhibition surrounding cylinders containing penicillin. The results were interpreted as evidence of a threshold for—SH vs. S-S groups or for dienols vs. diketones in the uninhibited and inhibited areas. It has been known for some time that—SH groups are essential metabolites for *Staphylococcus aureus* and that the blocking of —SH groups inhibits the growth of the staphylococci (Fildes, 1940). Our experimental data from cylinder plate assays, therefore, suggested that penicillin may act through this mechanism, i.e., by blocking —SH groups, thus lending biological support to the chemical evidence presented by Cavallito (1946).

We have also observed (unpublished experiments) that after exposure to bacteriostatic concentrations of penicillin cells of *S. aureus* are no longer gram-positive. This result is consistent with the findings of Henry and Stacey (1946) concerning the significance of the —SH group in the gram-positive complex. Further evidence that —SH groups may be involved in the interference of penicillin with the growth of *S. aureus* can be marshaled from the observation of Gale and Taylor (1946) that penicillin prevents the assimilation of glutamic acid, one of the constituents of glutathione.

In the present paper discussion is confined to phenomena that are revealed by staining and that may be considered to be incidental to changes affecting the sulfhydryl compounds. Active absorption of solutes by living cells may be assumed to entail expenditures of energy provided for by aerobic respiration which depends upon the cytochemical integrity of liponucleoproteins involving —SH. Therefore, interference of penicillin with —SH components of the respiratory systems might be postulated to effect (1) changes in the rate of absorption of solutes, and (2) swelling of the organisms coincident with the disorganization of the liponucleoproteins and liberation of lipids and fatty acids.

The results of our experiments tend to support these hypotheses. Our evidence obtained from vital staining of cells of *S. aureus in situ* on the assay plates or after transfer from different regions of the plates to a drop of the dye solution on a slide shows that, following exposure to bacteriostatic concentrations of penicillin, the cells lose their ability to accumulate neutral red, methyl green, or methylene blue within the vacuolar solution, and that they swell to at least twice their original diameter. Evidence was also obtained that concomitant

<sup>3</sup> Sold by National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, New York.

with or subsequent to the swelling of the cells, fatty acids appear. These may account for the downward shift of pH revealed by the use of indicators (Dufrenoy and Pratt, 1947).

The cytological observations reported in this paper are mainly from plates seeded and preincubated for 3 hours on which penicillin was subsequently allowed to diffuse during a 3-hour secondary incubation period, since in that short time bacteriostatic effects were obtained without extensive bacteriolysis in the inhibition zones. The standard 16-hour plates were not used routinely for the cytological observations, since, because of the extensive bacteriolysis that occurred in that length of time, it was difficult to find material suitable for study. The 3-hour technique described above more readily provided cells appropriate for our different studies. The longer diffusion period, on the other hand, was found to provide the best material for the study of lipids and fatty acids arising from bacteriolysis.

#### SUMMARY

In continuation of work previously reported, studies of penicillin assay plates have been made by means of techniques intended to delineate the cytochemical picture that develops on such plates when the test organisms are subjected to the action of penicillin.

The present paper concerns cytochemical changes that occur in different parts of bacterial cells exposed to bacteriostatic or bactericidal concentrations of penicillin.

The first evidence of the effect of penicillin on *Staphylococcus aureus* was observed to be the failure of the dividing organism to apportion vacuolar material to daughter cells.

This was followed by failure of the vacuoles to retain material normally encompassed therein.

These changes were manifest in cells under the influence of penicillin, first by loss of the ability to accumulate vital dyes in the vacuolar solution, and second by dispersion of the vacuolar solution, originally located in a central body, toward the periphery of the swelling cell.

This results, in such cells, in diffuse staining with vital dyes, with a relatively high concentration of the dye at the periphery of each cell.

The use of appropriate reagents showed that lipids are displaced from cells undergoing lysis under the influence of penicillin and that the liberated lipids are hydrolyzed into fatty acids.

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# MICROBIC DECOMPOSITION OF PANTOTHENIC ACID<sup>1</sup>

WILLIAM I. METZGER<sup>2</sup>

*Department of Bacteriology and Public Health, University of Illinois, College of Medicine, Chicago 12, Illinois*

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The role of vitamins in the nutrition of microorganisms is well known. In many cases the accessory growth factor functions as a coenzyme, apparently being little affected itself in the process. In direct contrast to this function is the fact that at least some of the vitamins may be attacked and decomposed (either partially or completely) by certain microorganisms. In this case the vitamin serves as a substrate for the particular enzymes of the cell involved, the resulting decomposition yielding energy and materials for possible use by the cell in its various metabolic activities. Relatively little is known regarding the dissimilation of accessory growth factors, but that such can occur is not surprising in view of the wide variety of organic compounds that are subject to the action of microorganisms. Certainly, such information would lead to a better understanding of the metabolism of these vital substances, especially their biosynthesis. The literature on this subject has been reviewed by Koser and Baird (1944).

The present work was undertaken with the thought that information concerning the metabolism of pantothenic acid could be gained if it were possible to find microorganisms capable of decomposing this substance. Such microorganisms were found in soil and air (belonging to the genus *Pseudomonas*), and their action on pantothenic acid is described.

## EXPERIMENTAL PROCEDURES

*Media and methods.* The experimental work resolved itself into two main portions, one being concerned with the decomposition of pantothenic acid in cultures of growing organisms and the other with decomposition of this substance by resting or washed cells. In the former, a simple medium containing pantothenate as the only carbon source was used extensively. This medium, containing the basal salt mixture of Koser and Baird (1944), had the following composition:

(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> .....	2.0 g
KH <sub>2</sub> PO <sub>4</sub> .....	1.5 g
NaCl.....	5.0 g
MgSO <sub>4</sub> .....	0.1 g
Pantothenate.....	0.1 to 1.0 g*
Distilled H <sub>2</sub> O.....	1,000 ml

\* Calcium pantothenate was commonly used in a concentration of 0.01 per cent because of the increasingly heavy precipitate formed with larger amounts. Sodium pantothenate<sup>3</sup> was used in a concentration of 0.1 per cent.

<sup>1</sup> Abstract of thesis presented in partial fulfillment of requirements for the Ph.D. degree.

<sup>2</sup> Present address: Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

<sup>3</sup> Kindly supplied by Dr. A. C. Bratton, Jr., Parke, Davis and Company, Detroit, Michigan.

The pH of the medium was adjusted to 7.5 to 7.8 and autoclaved at 15 pounds' pressure for 10 minutes. This medium will be referred to hereafter as pantothenate broth, the corresponding agar (pantothenate agar) being prepared by adding 1.5 per cent agar to the broth.

Results involving turbidimetric measurements were obtained with a lumetron model 400 G photoelectric colorimeter (wave length 530 m $\mu$ ).

Manometric experiments were carried out in the conventional manner, using the Warburg technique. Duplicate vessels, maintained at 30 C during experiments, were used in all cases, and to each were added 1.0 ml of M/20 phosphate buffer at pH 7.7, 1.0 ml of washed cells, and 0.5 ml of the substrate (in the side arm), in addition to KOH or H<sub>2</sub>SO<sub>4</sub>.

Resting cells were obtained by growing the organisms on 0.1 per cent pantothenate plus 0.2 per cent asparagine agar (asparagine increased the yield of cells while not affecting pantothenate utilization) in Kollé bottles for 48 hours at 33 C. The resultant growth was then washed from the agar with M/60 buffer (in most cases a phosphate buffer at pH 7.7), and the suspension was filtered through a thin layer of glass wool and centrifuged. After a second centrifugation in graduated centrifuge tubes, the packed cells were diluted 1:30 with the M/60 buffer, and this suspension was standardized for each experiment in the lumetron colorimeter. The various substrate solutions were carefully prepared and kept in the frozen state until used.

*Isolation and identification of organisms.* Various samples of soil were assayed for their content of pantothenate-utilizing organisms by means of two enrichment methods. The methods were essentially the same, except in one case pantothenate was added, at intervals, to moist soil, and in the other a small amount of soil was added to pantothenate broth. In both cases, repeated subcultures in pantothenate broth, combined with platings on pantothenate agar, tended to eliminate nonutilizing organisms, at the same time yielding a total of six pure cultures of bacteria that were capable of continued growth in pantothenate broth. These organisms were considered to be pantothenate utilizers (they showed little or no growth in the same medium without pantothenate) and were designated, according to their isolation numbers, as cultures 135, 401, 512, 513, 701, and 702. Three additional bacteria that could utilize pantothenate were obtained by allowing flasks of pantothenate broth to stand open in a laboratory room for several days. These were designated as cultures 2, 8, and 11.

All nine cultures were periodically checked for purity, were transferred weekly in pantothenate broth and onto pantothenate agar slants, and were kept at 33 C at all times. Reserve supplies of active and dried cultures were also maintained.

Attempts to isolate thermophilic pantothenate-utilizing organisms failed.

Partial identification of these organisms was accomplished. All of the cultures were strikingly alike in all of their characteristics: they were gram-negative, aerobic, motile, nonsporeforming, nonpigmented, nongranular, short to medium rods; they utilized glucose, sucrose, maltose, lactose, and mannite without acid



or gas; they were indole-negative and methyl-red- and Voges-Proskauer-negative; they were non-gelatin-liquefying; they reduced litmus milk with an alkaline reaction; they produced  $H_2S$  in small amounts; and six cultures reduced nitrate (five to nitrites and one to free nitrogen). Nutrient agar plate colonies were smooth, shiny, and grayish in color. These characteristics tend to place these organisms in the family *Pseudomonadaceae* (see references by Koser and Baird, 1944; Monias, 1928; Bergey *et al.*, 1939). Flagellar stains of cultures 11 and 512, with which most of the experimental work was done, showed that both had polar flagella only (Leifson's BBL flagellar stain). These organisms, therefore, have

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		25 C	33 C	33 C
	<i>hours</i>			
11	0	100,000	80,000	15,000
	24	920,000	3,200,000	20,000
	48	23,000,000	19,600,000	35,000
	72	491,000,000	403,000,000	43,000
	96	500,000,000	480,000,000	50,000
	144	450,000,000	325,000,000	47,000
512	0	207,000	312,000	50,000
	24	1,980,000	950,000	65,000
	48	42,600,000	75,000,000	108,000
	72	520,000,000	606,000,000	121,000
	96	783,000,000	537,000,000	135,000
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#### RESULTS OF EXPERIMENTS WITH GROWING CELLS

*Growth in pantothenate broth.* The effect of certain factors on the growth of *Pseudomonas* sp. in pantothenate broth was determined, mainly to arrive at optimum conditions for later work.

From table 1 showing the nutrient agar plate counts obtained with two typical cultures, it is obvious that temperature did not have much influence on growth in calcium pantothenate broth in the range of 25 to 33 C. Also, a prolonged lag phase was exhibited in this medium, rapid multiplication occurring only after 24 hours and continuing up to 72 hours. Little or no growth took place in the basal salt mixture without pantothenate, indicating that pantothenate was in fact being utilized for growth purposes. The low zero hour count in this medium was due to the fact that these tubes were inoculated from a subculture of the same medium to avoid carrying over pantothenate.

The pH of the medium was adjusted to 7.5 to 7.8 and autoclaved at 15 pounds' pressure for 10 minutes. This medium will be referred to hereafter as pantothenate broth, the corresponding agar (pantothenate agar) being prepared by adding 1.5 per cent agar to the broth.

Results involving turbidimetric measurements were obtained with a lumetron model 400 G photoelectric colorimeter (wave length 530 m $\mu$ ).

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The influence of pH on growth was determined by adjusting calcium pantothenate broth to various pH values (5.2, 5.5, 6.0, 6.5, 6.8, 7.2, 7.5, 7.7, and 8.0) with 0.5N HCl and 0.5N NaOH, the final concentration of pantothenate being 0.01 per cent in a total volume of 8.0 ml of medium. The incubation temperature was 33 C, and growth was determined every 24 hours with the lumetron colorimeter. The inoculum per experimental tube, which was the same in other experiments unless otherwise stated, was 0.1 ml of a 72-hour pantothenate broth culture incubated at 33 C. The results showed that all of the cultures responded similarly to variations in pH, the optimum for each being approximately 7.7. A quite rapid fall in turbidity occurred in the less alkaline media

TABLE 2

*Effect of pantothenate concentration on growth of Pseudomonas sp.*

CULTURE	CONCENTRATION OF PANTOTHENATE	TURBIDITY READINGS	
	$\mu\text{g/ml}$	3 days	7 days
11	0	98.5	97
	100	70	67
	500	63	61
	1,000	56	52
	5,000	58	54
	10,000	60	54
	100,000	73	63
512	0	96	94
	100	75	73
	500	67	65
	1,000	60	59
	5,000	63	60
	10,000	68	63
	100,000	79	66

(no growth took place below pH 5.5), and a slight decrease was observed also at pH 8.0. After 168 hours the pH of each medium was within 0.1 to 0.2 of a point of uninoculated controls, indicating that this factor (i.e., a change in pH during growth) would have no effect on growth.

To determine the optimum concentration of pantothenate for growth, sodium pantothenate in amounts of 0, 100, 500, 1,000, 5,000, 10,000, and 100,000  $\mu\text{g}$  per ml was added to the basal salt mixture, and turbidity readings were made after 3 days' incubation at 33 C and again after 7 days. It was found that 1,000  $\mu\text{g}$  per ml was optimum for both of the cultures studied, although 5,000  $\mu\text{g}$  per ml was practically as effective (table 2). Larger amounts of pantothenate, especially 100,000  $\mu\text{g}$  per ml, were definitely inhibitory to growth, although not completely so.

Another experiment showed that growth in pantothenate broth was the same whether the pantothenate was added to the medium before autoclaving or

whether a filtered solution of pantothenate was added aseptically to the autoclaved basal salt mixture. The addition of certain inorganic salts ( $\text{ZnCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{MnCl}_2$ , and  $\text{FeSO}_4$ ) did not improve nor impair growth in pantothenate broth.

Two analogues of pantothenic acid, pantoyltaurine<sup>4</sup> and *dl*-N-pantoyl-*n*-butylamine,<sup>5</sup> were studied for their effect on the growth of *Pseudomonas* sp. in pantothenate broth. It was found that the former could support growth when used in a concentration of 10,000  $\mu\text{g}$  per ml in the absence of pantothenate but not to the same extent as did the same concentration of pantothenate in the absence of pantoyltaurine. When both substances were present in the same medium, growth was considerably less than that in a medium which contained only pantothenate, unless the concentration of pantothenate was equal to or greater than the concentration of pantoyltaurine. Use of the same procedure with *dl*-N-pantoyl-*n*-butylamine, revealed that this substance (in a concentration of 10,000  $\mu\text{g}$  per ml) did not support growth, nor did added pantothenate cause growth unless an excess was present. With the reverse procedure (i.e., varying the concentration of the analogue and holding the pantothenate level constant) essentially the same relationship between these substances was noted. It is obvious that these results demonstrate a competitive type of inhibition.

*Growth in modified pantothenate broth.* To determine whether the components of pantothenic acid could be utilized for the growth of *Pseudomonas* sp., various media were made up as follows:  $\beta$ -alanine broth (40  $\mu\text{g}$  and 300  $\mu\text{g}$  per ml);  $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\gamma$ -butyrolactone<sup>6</sup> broth (60  $\mu\text{g}$  and 300  $\mu\text{g}$  per ml);  $\alpha$ , $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethyl-butyric acid (pantoic acid) broth (60  $\mu\text{g}$  and 300  $\mu\text{g}$  per ml); and various combinations of these media. Pantoic acid was prepared from the lactone according to the method of Sarett and Cheldelin (1945). These media were prepared as usual except that pantothenate was replaced by the compound or compounds under study in the concentrations indicated. All nine cultures were used in these tests, and the results were obtained by visual observations of turbidity. It was found that both  $\beta$ -alanine and pantoic acid supported growth, the amount of growth increasing in each case as the concentration of the substrate was increased. When combined, these substances gave growth approximately equal to that in control tubes of pantothenate broth. In no instance did the lactone support growth of any of the cultures, nor did it increase the effectiveness of  $\beta$ -alanine broth. It seems obvious that these organisms were unable to break the lactone ring, but when this structure is ruptured (as in pantoic acid) the compound could be utilized.

In another experiment, it was found that growth in pantothenate broth was not affected by removing  $(\text{NH}_4)_2\text{HPO}_4$  from the basal salt mixture. By also excluding atmospheric nitrogen, it was apparent that these organisms could

<sup>4</sup> Kindly supplied by Dr. F. A. Robinson, The Glaxo Laboratories, Ltd., Greensford, Middlesex, England.

<sup>5</sup> Kindly supplied by Dr. William Shive, University of Texas, Austin, Texas.

<sup>6</sup> Kindly supplied by Dr. D. F. Robertson, Merck and Company, Inc., Rahway, New Jersey.

deaminate  $\beta$ -alanine as a source of nitrogen. The salt was retained, however, as an extra nitrogen supply.

The effect of added nutrients upon the growth of *Pseudomonas* sp. in pantothenate broth was studied in order to try to improve the yield of bacteria for future work involving washed cells. Difco asparagine (0.2, 0.4, and 0.6 per cent) and smaco acid-hydrolyzed casein (0.5 per cent) each greatly improved growth of five of the cultures tested, as determined by visual turbidity. As was shown next, however, casein hydrolyzate "spared" the pantothenate, to a large extent, from being acted upon, whereas asparagine did not.

*Destruction of pantothenate during bacterial growth.* In order to prove that pantothenate was actually being decomposed during growth of these *Pseudomonas* cultures and to determine the rate of this decomposition, microbiological assays for pantothenate were carried out by the method of Skeggs and Wright (1944). With 0.1 per cent sodium pantothenate broth, it was found that all nine of the cultures had destroyed 100 per cent of the substrate within a growth period of 72 hours at 33 C. After 24 hours, cultures 11 and 512 (the only ones tested) had destroyed 20 per cent of the pantothenate.

To determine the effect of added nutrients upon the destruction of pantothenate, 0.2 per cent asparagine and 0.5 per cent casein hydrolyzate were added, respectively, to pantothenate broth. The media were inoculated with cultures 11 and 512, and the contents of each were assayed for their pantothenate concentration after 24 and 72 hours' incubation at 33 C. The results with asparagine were the same as those obtained in its absence, but with casein hydrolyzate no breakdown was detected with either culture at 24 hours, and at 72 hours less than 50 per cent decomposition of pantothenate had occurred. Therefore, the use of casein hydrolyzate was discontinued, but asparagine was later incorporated in pantothenate agar for the production of washed cells.

*Survey of other microorganisms for pantothenate utilization.* It was of interest to determine whether various representative stock cultures of bacteria or fungi possessed any native ability to decompose pantothenic acid. The methods used seemingly afforded the microorganisms optimum conditions for attacking pantothenate, but in all cases the results were negative. Therefore, for brevity, the details of this work will not be described. It is reasonable to assume, however, that in nature many of these same microorganisms may play an active role in decomposing pantothenate, having lost this function on repeated transfer in the laboratory.

#### RESULTS OF EXPERIMENTS WITH RESTING CELLS

*Deamination and Thunberg studies.* An investigation into the deamination of pantothenate was performed by adding 1.0 ml of washed cells (preparation previously explained), 1.0 ml of M/20 phosphate buffer at pH 7.5, and 3.0 ml of M/50 substrate to duplicate test tubes and determining ammonia production with Nessler's reagent after incubation periods of 2, 6, and 24 hours at 33 C. It was found that pantothenate was deaminated and that the reaction progressively increased from 2 to 24 hours, meaning that the  $\beta$ -alanine portion

of pantothenic acid was actually the substance being acted upon. A similar experiment with  $\beta$ -alanine did, in fact, give the same results as were obtained with pantothenate. Also, similar results were obtained with *dl*-alanine, this substance being included to determine the specificity of the deaminase. These findings are in agreement with other reports (Stephenson, 1939) that *pseudomonae* can deaminate various amino acids.

Preliminary to carrying out manometric experiments, it was of interest to determine whether a representative culture, culture 11, could reduce methylene blue with  $\beta$ -alanine, pantoic acid, pantoyl-lactone, and pantothenate as substrates. The usual Thunberg technique was used, in which 1.0 ml of washed

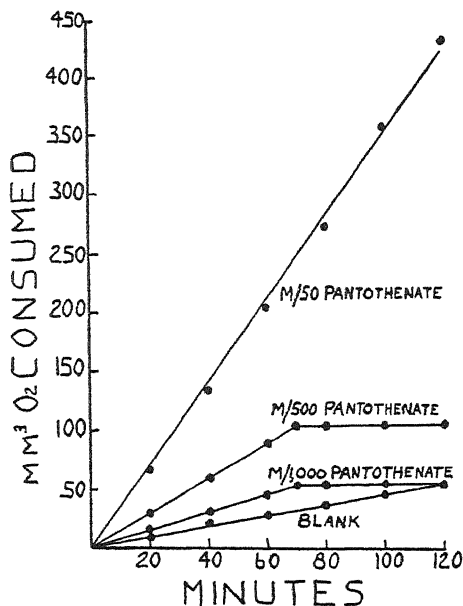


FIG. 1. THE OXIDATION OF PANTOTHENATE BY *PSEUDOMONAS* SP., CULTURE 11

cells, 1.0 ml of M/20 phosphate buffer at pH 7.5, 0.5 ml of 1:10,000 methylene blue, and 0.5 ml of M/50 substrate were placed in duplicate Thunberg tubes, the tubes evacuated, the cells tipped in from the side arm at the zero time, and the tubes incubated at 33 C and observed visually at 5-minute intervals for decolorization. It was found that the methylene blue was completely decolorized in 65 minutes with pantothenate as the substrate, in 80 minutes with  $\beta$ -alanine, and in 75 minutes with pantoic acid. Tubes containing the lactone remained blue for a much longer period of time, decolorizing at the same rate as the controls.

*Warburg studies; oxidation of pantothenate.* From the foregoing experiment, it was expected that pantothenate would be readily oxidized in the Warburg apparatus. This was found to be true, and the oxidation of various concentrations of pantothenate by culture 11 is shown in figure 1. It is apparent that

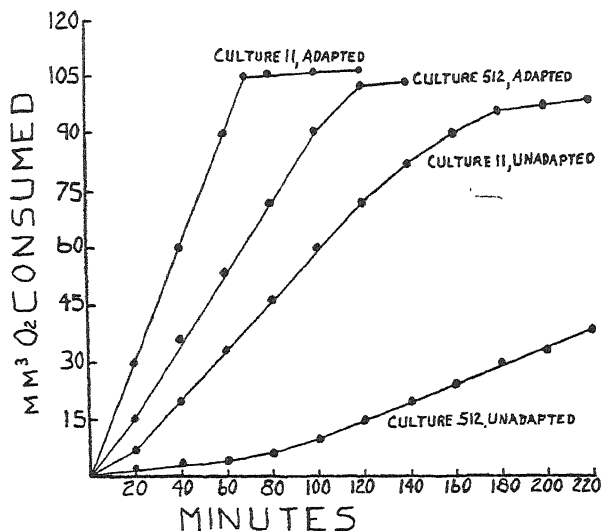


FIG. 2. THE OXIDATION OF  $M/500$  PANTOTHENATE BY ADAPTED AND UNADAPTED CELLS OF *PSEUDOMONAS* SP.

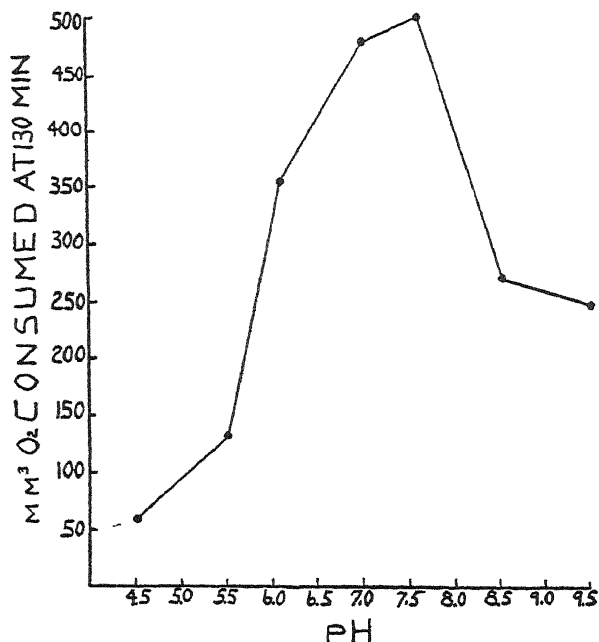


FIG. 3. THE EFFECT OF pH ON THE OXIDATION OF  $M/50$  PANTOTHENATE BY *PSEUDOMONAS* SP., CULTURE 11

the reaction with  $M/500$  and  $M/1,000$  substrates reaches completion in approximately 70 minutes, at which time about 50 per cent of the total oxygen (theo-



retical) needed for complete combustion has been used. Although carbon dioxide curves were not established, it was found that after 70 minutes a total of 90 mm<sup>3</sup> of this gas had been evolved from m/500 pantothenate, this being equivalent to 4 molecules of carbon dioxide. Since the oxygen consumption of this substrate was equal to 5 molecules, the respiratory quotient (R.Q.) at 50 per cent oxidation was 0.80 as against a theoretical R.Q. of 0.90 for complete combustion.

Cultures 512 and 702 required 120 and 200 minutes, respectively, to achieve 50 per cent oxidation of m/500 pantothenate, but it is noteworthy that they, too, eventually completed the reaction at the same end point as did culture 11. Of the three, only culture 702 exhibited any significant lag period at the beginning of the reaction. The endogenous respiration, especially of culture 11, was rather high, but attempts to lower it, such as aerating the cells for 1 hour prior to use, were not successful. In all cases corrections for the blank were made since it has not been definitely established that endogenous respiration is suppressed in the presence of a readily utilizable substrate, although such may be the case.

The adaptive nature (Dubos, 1940) of the enzymes involved was shown by the fact that 48-hour nutrient agar cultures (unadapted) were much less active than were adapted cultures which had been maintained on pantothenate agar (figure 2). Three transfers of the unadapted cultures on pantothenate-asparagine agar, however, fully adapted them for the utilization of pantothenate.

A study into some of the factors which might influence the oxidation of pantothenate revealed that the optimum pH was around 7.7 (figure 3). m/50 substrate was used in this experiment so that any differences in activity at the various pH levels would be magnified. An intensive study into the effect of temperature on the oxidation of pantothenate was not made, but it was found that an increase to 38 C (all experiments were conducted at 30 C) neither affected the rate nor the degree of the reaction. It was also found that the enzymes involved in the oxidation were stable for at least 10 days when stored (in the form of packed cells) in the refrigerator (not frozen). Thus, washed cells were usually prepared a day before use and stored in the packed state overnight in the refrigerator. Also, it was revealed that physiologically young cells (48 hours) were much more active than 96-hour (or older) cells. This was expected but, unfortunately, owing to insufficient yield, 24-hour cells could not be used.

Various attempts to explain the incomplete oxidation of pantothenate were unsuccessful and, without going into the details of this work, it may be said that the only logical explanation for this phenomenon was that a reaction of oxidative assimilation was occurring, the nature of which will be briefly discussed later.

A survey of four nutrient agar stock cultures of bacteria, namely *Escherichia coli-communior*, *Proteus vulgaris*, *Acetobacter suboxydans*, and *Pseudomonas aeruginosa* showed that the first three had absolutely no activity on m/50 pantothenate, and *P. aeruginosa* had only a very minimal activity, which was not increased by three transfers on pantothenate-asparagine agar.

Experiments with pantothenate analogues showed that m/50 dl-N-pantoyl-n-

butylamine was not oxidized and, moreover, did not interfere with the oxidation of M/500 pantothenate when both were present in the same vessel. Apparently this substance inhibits growth by some mechanism other than an interference with pantothenate utilization. Pantoyltaurine was oxidized to some extent but the taurine component was not, indicating that taurine may interfere with the metabolism of pantoic acid as well as that of  $\beta$ -alanine, since pantoyltaurine was not oxidized to the same extent as was pantoic acid alone.

*Oxidation of  $\beta$ -alanine and pantoic acid.* Figure 4 shows the oxidation of M/50 and M/500  $\beta$ -alanine and M/500 pantoic acid by culture 11. The reaction with both components was completed in 100 minutes and, by appropriate calculations, it was determined that M/500  $\beta$ -alanine was oxidized to two-thirds of completion and M/500 pantoic acid to approximately 43 per cent of completion, 2 and 3 molecules of oxygen having been consumed, respectively. The decided

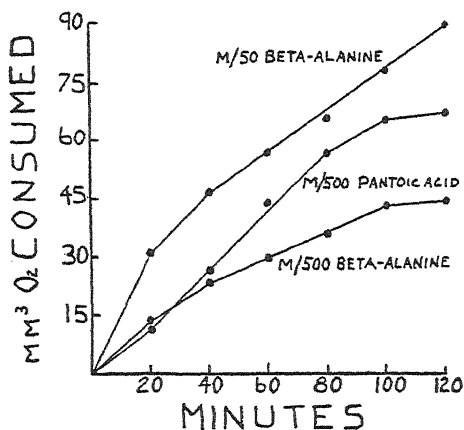


FIG. 4. THE OXIDATION OF  $\beta$ -ALANINE AND PANTOIC ACID BY *PSEUDOMONAS* SP., CULTURE 11

break in the oxidation rate of  $\beta$ -alanine after 20 minutes is impossible to explain (M/1,000 substrate, not shown—presented the same type of curve). After 100 minutes, 2 molecules of carbon dioxide had been evolved from M/500  $\beta$ -alanine and a like number from pantoic acid, giving respective R.Q. values of 1.0 and 0.67 as against theoretical R.Q. values of 1.0 and 0.86, respectively, for complete oxidation. Results similar to those obtained for  $\beta$ -alanine were reported by Webster and Bernheim (1936) for *dl*-alanine, using *Pseudomonas aeruginosa*. When both  $\beta$ -alanine and pantoic acid were present in the same vessel, the oxidative curve was similar to that obtained with an equivalent amount of pantothenate. The lactone moiety of pantothenic acid was not oxidized by culture 11, using M/50 and M/500 concentrations.

*Experiments with cell poisons.* As noted earlier, the incomplete oxidation of pantothenate suggested a type of reaction described many times before (Clifton, 1946) and called oxidative assimilation. Briefly, the reaction is one in which the substrate, under normal conditions, is not completely oxidized, but

instead a substance, or substances, with the empirical formula of a carbohydrate is formed. This substance, then, is assimilated by the cells, preventing any further degradation. The addition of an appropriate enzyme inhibitor, however, prevents the processes of assimilation from continuing, allowing dissimilation to proceed.

With KCN as the inhibitor of assimilation in this case, it was found that the oxidation of M/500 pantothenate was greatly increased in the presence of certain

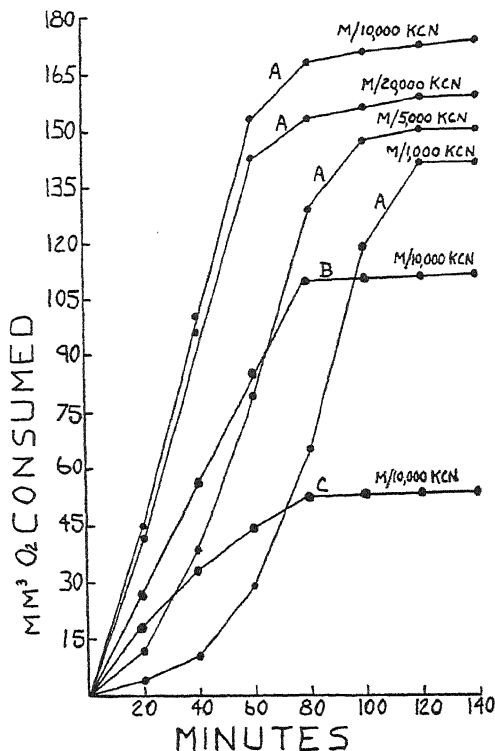
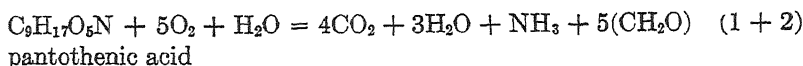
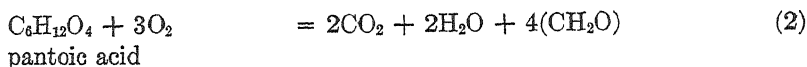
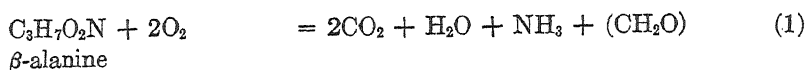


FIG. 5. THE EFFECT OF KCN ON THE OXIDATION OF M/500 PANTOTHENATE (A), M/500 PANTOIC ACID (B) AND M/500  $\beta$ -ALANINE (C) BY *PSEUDOMONAS* SP., CULTURE 11

concentrations of cyanide (figure 5), the reaction proceeding to 78 per cent of completion with M/10,000 KCN. Similar results were obtained with  $\beta$ -alanine and pantoic acid, the former going to approximately 80 per cent of completion and the latter to approximately 71 per cent of completion in the presence of M/10,000 KCN (figure 5). In each case carbon dioxide evolution also increased, the R.Q. values more nearly approaching those for complete dissimilation of each respective substrate (table 3).

These results leave no doubts that the normal oxidation of pantothenate (and its components) is one of oxidative assimilation. Therefore, the normal

oxidation of these substances may be represented by the following balanced equations:



Attempts to show a 100 per cent decomposition of pantothenate with other enzyme inhibitors were unsuccessful. Monoiodoacetic acid (M/5,000 and M/50,000) greatly inhibited the oxidation of M/500 pantothenate, whereas sodium azide gave results very similar to those obtained with KCN, but in different concentrations. Endogenous respiration was not significantly affected by any of the inhibitors.

TABLE 3

*Comparative results of oxygen consumption and carbon dioxide evolution with and without potassium cyanide (M/10,000)*

SUBSTRATE	OXYGEN				CARBON DIOXIDE				R.Q.	
	No KCN		With KCN		No KCN		With KCN		No KCN	With KCN
	mm <sup>3</sup>	mol.	mm <sup>3</sup>	mol.	mm <sup>3</sup>	mol.	mm <sup>3</sup>	mol.		
M/500 Pantothenate...	110	5	175	7.8	90	4	146	6.5	0.80	0.83
M/500 $\beta$ -Alanine.....	45	2	54	2.4	45	2	54	2.4	1.0	1.0
M/500 Pantoic acid....	66	3	112	5.0	45	2	88	3.9	0.67	0.78

## DISCUSSION

The results of this study again tend to emphasize the microbial decomposition of vitamins in contrast to their usual role as accessory growth factors. It is striking that most of the studies of this nature have been done with pseudomonae and of significance that in each case the organisms were isolated directly from soil or other natural habitats, undoubtedly involving a process of natural adaptation. What importance these studies have in relation to the decomposition of vitamins in the human intestinal tract is unknown.

In the present study it was found that the lactone moiety of pantothenic acid could neither serve as a growth substrate nor as an oxidizable substrate for *Pseudomonas* sp., whereas its hydrolyzed counterpart, pantoic acid, was readily utilized by both growing and resting cells. This is interesting in view of the fact that Stansly and Schlosser (1945) reported that pantoic acid is more readily utilized than is the lactone for the synthesis of pantothenic acid by *Escherichia coli*. They stated that pantoic acid is the probable precursor in the biological synthesis of pantothenic acid, rather than pantolactone.

Although the evidence points undeniably to a process of oxidative assimilation in the decomposition of pantothenate, it is true that a carbohydrate has not been actually demonstrated. Giesberger (1936), in similar experiments, did show an increase in the volutin content of *Spirillum serpens*, but most investigators have not studied this particular problem in detail. Suffice it to say that, knowing all of the facts, no other logical explanation of the phenomenon is possible.

It is realized that actively proliferating cells may not act in the same manner on pantothenate as do resting cells, but no attempt was made here to determine this relationship. Whelton and Doudorff (1945), however, did show that both types of cells of *Pseudomonas saccharophila* assimilated some substrates in essentially the same manner and other substrates in quite a different manner.

From the results obtained here, it appears that the carbohydrate substance produced during the oxidation of pantothenate is formed with great economy by the bacterial cells, approximately 55 per cent of the carbon of the substrate being assimilated. In similar studies, Barker (1936) found that the alga, *Prototheca zopfii*, converts from 50 per cent to more than 80 per cent of the carbon of various substrates into a carbohydrate material. This undoubtedly explains the ability of some of these microorganisms to survive and multiply in simple media.

The manner in which bacterial cells form this carbohydrate from a substance such as pantothenic acid is not clearly evident, although Clifton and Logan (1939) have postulated a theory, from known facts, for the formation of carbohydrate from various substrates by cells of *Escherichia coli*. Presumably, the process represents more than a mere reduction of carbon dioxide. Equally intriguing is the manner in which a poison like KCN selectively blocks the assimilatory process.

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#### SUMMARY

Bacteria of the genus *Pseudomonas* have been isolated from soil and air that could utilize pantothenate as a growth substrate in a medium containing only pantothenate and inorganic salts.  $\beta$ -Alanine and pantoic acid also were utilized when substituted for pantothenate, but pantoyl-lactone did not support growth.

During their growth, these bacteria decomposed 20 per cent of the pantothenate in 0.1 per cent pantothenate broth within 24 hours and 100 per cent of the substrate within 72 hours.

Pantoyltaurine and *dl*-N-pantoyl-*n*-butylamine, analogues of pantothenic acid, exhibited a competitive type of inhibition with pantothenate but only the former was able to support growth when substituted for pantothenate, and then to a lesser extent.

Manometric studies showed that pantothenate,  $\beta$ -alanine, and pantoic acid were oxidized, respectively, to 50 per cent, 67 per cent, and 43 per cent of completion by a process of oxidative assimilation. Pantoyl-lactone was not oxidized.

Potassium cyanide and sodium azide, in critical concentrations, caused the

oxidation of pantothenate,  $\beta$ -alanine, and pantoic acid more nearly to reach completion, presumably by inhibiting the processes of assimilation.

*dl*-N-Pantoyl-*n*-butylamine was not oxidized and pantoyltaurine was only slightly oxidized in the Warburg apparatus.

Various stock cultures of bacteria and fungi were not able to utilize pantothenate as a carbon source, nor were they able to oxidize this substance.

The significance of these findings is discussed.

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# PRODUCTION OF MOLD AMYLASES IN SUBMERGED CULTURE

E. H. LE MENSE,<sup>1</sup> JULIAN CORMAN, J. M. VAN LANEN<sup>2</sup>,  
AND A. F. LANGLYKKE

*Fermentation Division, Northern Regional Research Laboratory<sup>3</sup> Peoria, Illinois*

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During recent years many new and extended uses have been developed for fungal enzymes. Prominent among these are applications in the fields of food manufacturing, textile processing, and in the manufacture of malt beverages and industrial alcohol.

Although certain microorganisms are capable of elaborating amylases when grown under submerged conditions either aerobically (Waldmann, 1942) or anaerobically (Hockenhull and Herbert, 1945), industrial production methods generally involve cultivation on the surface of unagitated liquid or semisolid substrates. Exceptions are the "amylo" process (Owen, 1933) and a modified amylo process (Erb and Hildebrandt, 1946), in which selected strains of *Rhizopus* or *Mucor* are grown under submerged, aerobic conditions to saccharify grain mashes prior to alcoholic fermentation. More commonly, as in the production of mold bran (Underkofler, *et al.*, 1939; Boyer and Underkofler, 1945) and bacterial amylases (Beckord *et al.*, 1945, 1946), media are incubated in shallow layers in closed vessels or in open trays. Attempts to adapt these microorganisms to deep tank conditions to produce comparable yields of amylase have been unsuccessful.

The submerged culture method of producing amylases would have definite advantages when the product could be employed directly without concentration or purification as, for example, in the alcoholic fermentation of grain and in the manufacture of sugars and dextrans from starch. With these applications in mind a survey was made of a large number of molds to determine their ability to synthesize starch-hydrolyzing enzymes when cultured under submerged conditions. The present report deals with (1) the results of this survey of fungi, (2) the factors affecting the elaboration of amylases by promising strains, and (3) the substitution of mold amylase thus produced for distillers' malt. Pilot plant studies have been conducted with some of the promising strains disclosed herein, and the results of these experiments will be reported at a later date.

## METHODS

*Culture survey.* The cultures investigated were selected from the culture collection of the Northern Regional Research Laboratory. The basal medium for the survey of cultures was thin stillage obtained from the alcoholic fermentation

<sup>1</sup> Present address: Mold Bran Company, Eagle Grove, Iowa.

<sup>2</sup> Present address: Hiram Walker and Sons, Inc., Peoria, Illinois

<sup>3</sup> One of the four regional laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

of corn and sorghums. It contained 4 to 5 per cent of dry substance, approximately one-third of which was protein ( $N \times 6.25$ ). To favor the growth of all the organisms studied, 2 per cent of glucose and 0.5 per cent of calcium carbonate were added to the stillage. This medium was sterilized with steam at 20 p.s.i. gauge for 30 minutes.

For evaluation of the selected organisms for amylase production, cultures were grown first in 50 ml of thin stillage medium contained in 200-ml flasks. After 24 hours' incubation, 10 ml of culture were transferred to 200 ml of the supplemented stillage medium contained in 1-liter flasks. All cultures were incubated at 30 C and were shaken continuously at 90 three-inch strokes per minute in a Kahn type shaker. Samples were removed periodically for the determination of amylase activity.

Culture liquors were analyzed for the presence of dextrinizing enzyme by the method of Sandstedt *et al.* (1939) as modified by Olson, Evans, and Dickson (1947). Units of dextrinizing enzyme reported herein are the grams of soluble starch (Merck, Lintner) which, in the presence of excess *beta*-amylase, are dextrinized in 1 hour at 20 C.<sup>4</sup>

*Variations of cultural conditions.* To determine the influence of different carbohydrate and protein sources on amylase production, a fungal strain which was found to possess exceptional amylolytic activity was grown under conditions identical to those obtaining in the survey except that various protein and carbohydrate materials were substituted for thin stillage and glucose.

A study of the effects of different concentrations of calcium carbonate and calcium chloride was made under the same cultural conditions used in the survey except that 2 per cent of ground corn was substituted for 2 per cent of glucose.

Aeration rate studies were conducted on a somewhat larger scale than were the aforementioned experiments; that is, 4-liter quantities of stillage medium supplemented with 2 per cent of corn meal and 0.5 per cent of calcium carbonate were dispensed in 8-liter pyrex cylinders equipped with lids of aluminum plate and with air spargers of perforated aluminum tubing. These were sterilized with steam at a pressure of 25 p.s.i. gauge for 1 hour, cooled, and inoculated with 5 per cent by volume of a 24-hour culture. Air for use in the experiments was filtered through sterile cotton before introduction into the medium.

It was observed in the course of these experiments that strains which saccharified starch rapidly, and consequently were most suitable as replacements for barley malt, formed appreciable amounts of maltase. Following this observation, both dextrinizing and maltase activities of culture liquors were determined. Maltase activity was measured by determining the increase in reducing power by the method of Somogyi (1945) after incubating 10 ml of culture filtrate with 20 ml of a 1.05 per cent solution of maltose for 2 hours at 30 C. The enzyme-substrate mixture was maintained at a pH of 4.6 by the addition of acetate buffer to the maltose solution.

<sup>4</sup> A recent collaborative study of the *alpha*-amylase values of experimentally produced barley malts in which this method was used showed a range of activity from 9.8 to 30.5 units per gram. Commercial distillers' malt contains in the neighborhood of 25 units per gram.



*Conversion and fermentation of grain mashes.* Cultures producing appreciable quantities of amylase were further evaluated by determining their ability to replace barley malt in the alcoholic fermentation of corn. Forty-nine and one-half g of ground corn and 0.5 g of ground barley malt were placed in 500-ml Erlenmeyer flasks, and 170 ml of tap water heated to 70 C were added. The flasks were placed in a 70 C water bath and the grain slurries were stirred intermittently for 30 minutes. The mashes thus premalted were then cooked in the autoclave at a steam pressure of 25 pounds for 30 minutes and cooled to 75 C; mold culture liquor was added, together with sufficient water to lower the temperature to 55 to 56 C. Conversion was continued at 55 to 56 C for 30 minutes, during which period the mashes were agitated frequently. The same procedure was followed with the control mashes saccharified with malt except that 45 g of corn and 5 g of barley malt were used, 0.5 g of malt again being used for pre-malting and 4.5 g for conversion. Converted mashes were cooled to 30 C and inoculated with 2 per cent by volume of a 24-hour culture of distillers' yeast, strain NRRL Y567. The final volume in each flask was approximately 250 ml. Fermentation was conducted at 30 C for 72 hours, during which time the flasks were weighed periodically. The beers were then brought to a volume of 300 ml and aliquots of 200 ml taken for the determination of alcohol. One hundred ml of distillate were collected from each aliquot, and the concentration of alcohol in the distillate was determined by measuring its refractive index.

#### EXPERIMENTAL RESULTS

The results of the survey of fungi for ability to produce amylase in supplemented thin stillage medium are presented in table 1. Of 80 cultures of *Penicillium*, representing 18 species, only 8 formed detectable quantities of dextrinizing enzyme. All of these were of relatively low activity, the best being a strain of *P. purpurogenum* which gave 0.6 units per ml.

Two hundred seventy-eight of the cultures that were studied belonged to the genus *Aspergillus* and represented 41 different species. Only 34 members of this group elaborated dextrinizing enzyme. The culture liquors from active organisms varied from 0.1 to 15.3 units per ml. Although there was considerable variation between strains within a species, a high percentage of strains of *A. oryzae*, *A. wentii*, and *A. niger* was active. *Aspergillus niger* NRRL 337 gave the highest potencies of any organisms tested in the survey. Under the most favorable conditions, potencies up to 22.5 units per ml were obtained with it. On a dry basis (culture liquors contained about 2 per cent of solids) such preparations would have a potency of 1,125 units per gram.

Subsequently, *Aspergillus niger* strains NRRL 326, 330, and 679 were found to elaborate an enzyme complex which rapidly saccharified starch, although the formation of dextrinizing enzyme was not so marked as with *A. niger* NRRL 337.

When strains of *Rhizopus*, *Mucor*, and *Monilia* were grown under the same conditions, little or no dextrinizing enzyme was produced, although excellent growth was obtained. Despite their apparent lack of dextrinizing enzyme, culture liquors from a strain of *Rhizopus* NRRL 1891 received under the label "Rhizopus 'Boulard,'" were capable of considerable saccharification of grain

TABLE 1

*The production of amylase by various fungi grown submerged in thin stillage medium*

GENUS	CULTURES TESTED	NUMBER ACTIVE*	ACTIVE CULTURES		CONCENTRATION OF DEXTRINIZING ENZYME PRODUCED
			species	NRRL no.	units/ml
<i>Penicillium</i>	80	8	<i>P. urticae</i>	991	0.1
			<i>P. roseo-citream</i>	889	0.1
			<i>P. spiculosporum</i>	1027	0.2
			<i>P. chlorophaeum</i>	816	0.2
			<i>P. citreo-roseum</i>	835	0.2
			<i>P. aurantio-griseum</i>	972	0.2
			<i>P. brunneo-rubrum</i>	842	0.3
			<i>P. purpurogenum</i>	1064	0.6
<i>Aspergillus</i>	278	34	<i>A. versicolor</i>	231	0.1
			<i>A. candidus</i>	305	0.6
			<i>A. alliaceus</i>	315	1.7
			<i>A. foetidus</i>	341	1.3
			<i>A. niger</i>	622	0.1
			<i>A. niger</i>	624	0.1
			<i>A. niger</i>	606	0.1
			<i>A. niger</i>	617	0.1
			<i>A. niger</i>	607	0.1
			<i>A. niger</i>	605	0.1
			<i>A. niger</i>	614	0.1
			<i>A. niger</i>	354	0.4
			<i>A. niger</i>	679	1.1
			<i>A. niger</i>	326	2.2
			<i>A. niger</i>	330	6.0
			<i>A. niger</i>	337	15.3
			<i>A. niger</i>	363	1.6
			<i>A. wentii</i>	382	0.1
			<i>A. wentii</i>	378	0.1
			<i>A. wentii</i>	1207	0.2
			<i>A. wentii</i>	381	0.3
			<i>A. wentii</i>	1778	0.4
			<i>A. wentii</i>	377	0.4
			<i>A. wentii</i>	1269	0.6
			<i>A. oryzae</i>	480	0.1
			<i>A. oryzae</i>	474	0.1
			<i>A. oryzae</i>	464	1.2
			<i>A. oryzae</i>	449	2.9
			<i>A. oryzae</i>	694	3.1
			<i>A. oryzae</i>	698	3.3
			<i>A. oryzae</i>	454	3.0
			<i>A. flavus</i>	488	0.1
			<i>A. flavus</i>	491	0.2
			<i>A. gymnosardae</i>	505	0.2
<i>Rhizopus</i>	5	none			
<i>Mucor</i>	3	none			
<i>Monilia</i>	1	none			

\* Cultures were termed active if the  $\alpha$ -amylase activity obtained in their filtrates equaled or exceeded 0.1 unit per ml.

mashes (table 6). Other species of *Rhizopus* and *Mucor* were similar in behavior, suggesting that these organisms have amylolytic enzyme systems different from barley malt and the other molds examined in this study. In contrast to this observation, Leopold and Starbanow (1943) have reported the production of both  $\alpha$ - and  $\beta$ -type amylolytic enzymes by *R. japonicus*.

TABLE 2

*The production of dextrinizing enzyme by Aspergillus niger NRRL 337 cultivated in various media*

PROTEIN SOURCE	CARBOHYDRATE SOURCE	CONCENTRATION OF DEXTRINIZING ENZYME
		units/ml
Corn steep liquor, 3%	None	2.2
Corn steep liquor, 3%	Glucose, 2%	8.2
Corn steep liquor, 3%	Molasses, 2%	4.6
Corn steep liquor, 3%	Corn meal, 2%	10.2 ✓
Dried tankage, 2%	None	2.1
Dried tankage, 2%	Glucose, 2%	9.3
Dried tankage, 2%	Molasses, 2%	11.5 ✓
Dried tankage, 2%	Corn meal, 2%	8.7
Soybean meal, 2%	None	7.9
Soybean meal, 2%	Glucose, 2%	7.4
Soybean meal, 2%	Molasses, 2%	8.5
Soybean meal, 2%	Corn meal, 2%	11.2 ✓
Thin stillage	None	1.7
Thin stillage	Glucose, 2%	11.5
Thin stillage	Molasses, 2%	7.9 ✓
Thin stillage	Corn meal, 2%	16.5 ✓
Thin stillage	Xylose, 2%	5.3
Thin stillage	Lactose, 2%	6.7
Thin stillage	Sucrose, 2%	11.0
Thin stillage	Maltose, 2%	14.5 ✓

Enzyme determinations were made after cultures were shaken for 5 days.

Composition of medium: Protein and carbohydrate as shown plus 0.5 per cent calcium carbonate.

*Factors affecting enzyme production by Aspergillus niger NRRL 337.* To determine whether nutrients other than those present in thin stillage were satisfactory for amylase production, media containing protein from several other sources were supplemented with various carbohydrates. Calcium carbonate was added to give a concentration of 0.5 per cent. After sterilization, the media were inoculated with 2 per cent by volume of a submerged culture of *Aspergillus niger* NRRL 337 and incubated, with continuous shaking, for 5 days. The results are shown in table 2.

Thin stillage, corn steep liquor, and animal tankage when not supplemented with carbohydrate gave low yields of amylase, but soybean meal appeared to be

satisfactory without added carbohydrate. When commercial glucose, molasses, or corn meal was added to the protein basal media, good amylase formation resulted except when corn steep liquor was supplemented with molasses. In this series of experiments the highest enzyme concentration (16.5 units per ml) was obtained with thin stillage to which corn meal was added. Sucrose and maltose gave good enzyme formation, whereas xylose and lactose were less effective when added to thin stillage. These results indicate that a wide variety of carbohydrates in conjunction with proteinaceous substances of animal and plant origin can be employed for the production of amylase by this organism.

The influence of calcium carbonate on amylase production is demonstrated in the following experiment, the results of which are shown in table 3. Calcium carbonate, in varying amounts to give concentrations ranging from 0 to 1.0 per

TABLE 3

*The effect of calcium carbonate and calcium chloride on the production of dextrinizing enzyme by Aspergillus niger NRRL 337*

SOURCE OF CALCIUM		FINAL pH	$\alpha$ -AMYLASE  units/ml
Salt added	Concentration  per cent		
None		4.0	1.5
CaCO <sub>3</sub>	0.10	4.3	7.9 ✓
CaCO <sub>3</sub>	0.25	4.9	8.9
CaCO <sub>3</sub>	0.50	5.3	9.2
CaCO <sub>3</sub>	1.00	5.4	8.5
CaCl <sub>2</sub>	1.00	3.7	1.2

Cultures were analyzed after an incubation period of 3 days at 30 C.

Composition of base medium: Distillers' thin stillage plus 2 per cent corn.

cent, was added to thin stillage containing 2 per cent ground corn. *Aspergillus niger* NRRL 337 was cultured in these media for 3 days, after which the culture liquors were analyzed for dextrinizing potency. Whereas the enzyme activity was low in the absence of calcium carbonate, the addition of 0.1 per cent calcium carbonate gave more than a 5-fold increase in dextrinizing power, that is, from 1.5 to 7.9 units per ml. The optimum concentration of calcium carbonate appeared to be in the neighborhood of 0.25 to 0.5 per cent, resulting in potencies of 8.9 and 9.2 units per ml, respectively. The pH of the fermented liquors ranged from 4.0 in media without calcium carbonate to 4.3 to 5.4 in those in which it was used. When calcium chloride at a concentration of 1 per cent was substituted for calcium carbonate, the final pH was 3.7, and the enzyme production was lower than that in the control without added calcium salt. Since it is well known that  $\alpha$ -amylase is readily inactivated at a pH of 4.2 or lower, it appears that the principal action of the calcium carbonate in stillage medium is to maintain the pH above this point during the fermentation. However, a specific stabilizing effect of the calcium ion upon mold dextrinizing amylase has been demonstrated (Nakamura, 1931), and this may have been a contributing factor in those instances in which the reaction was favorable to amylase stability.

The influence of aeration upon amylase production is shown in table 4. *Aspergillus niger* NRRL 337 was grown in supplemented thin stillage medium in glass cylinders, as previously described. The aeration rate was varied from 0.25 volumes to 1.0 volume of air per volume of medium per minute. Dextrinizing enzyme and pH were determined daily from the second through the seventh day. It was found that enzyme synthesis increased progressively with increased rates of aeration. With 0.25 volume of air the final potency of the liquor was 2.4 units per ml; with 0.5 volume of air, 9.0 units per ml; and with 1.0 volume of air, 22.5 units per ml. In larger fermentations in which media were both aerated and agitated, a lower rate of aeration was found to be adequate for maximum enzyme production (Le Mense *et al.*, 1947).

*Substitution of mold culture liquors for malt in alcoholic fermentations.* Culture liquors from the preceding experiment were investigated for their ability to replace barley malt in the saccharification of grain mashes for alcoholic fermentations. The liquors were used at levels of 8, 13, and 20 per cent of the final mash

TABLE 4

*The influence of the rate of aeration on the production of dextrinizing enzyme by Aspergillus niger NRRL 337*

AERATION RATE	DEXTRINIZING ENZYME AFTER					
	2 days	3 days	4 days	5 days	6 days	7 days
<i>L air/L medium/ minute</i>	<i>units/ml</i>					
0.25	0.6	1.4	1.8	2.7	2.2	2.4
0.5	4.8	6.9	7.9	8.2	9.0	9.0
1.0	6.2	8.9	12.9	15.8	22.0	22.5

volume. One-tenth of this amount in each instance was added as premalt. Corn was the only grain used in the mashes saccharified with mold amylase, whereas control mashes contained 90 per cent corn and 10 per cent barley malt, one-tenth of the malt also being employed as premalt. The results of these experiments are shown in table 5.

Malt-converted control mashes gave an average yield of 5.15 proof gallons of alcohol per bushel of grain. When used at a level of 13 per cent of the mash volume, culture liquors produced by aerating at 0.25 volume of air per volume of medium per minute for a 7-day culture period gave only 4.24 proof gallons of alcohol per bushel of grain. Culture preparations aerated at 0.5 volume per volume of medium per minute gave yields equivalent to or better than malt when used at 13 and 20 per cent levels after 4 days of incubation and when used at 8, 13, and 20 per cent levels after 7 days of incubation. Cultures aerated at 1 volume of air per volume of medium per minute were satisfactory in all cases except those to which a liquor cultured for 2 days was added at an 8 per cent level. The highest alcohol yields, amounting to 5.50 and 5.40 proof gallons per bushel, were obtained with 20 per cent levels of culture liquor aerated at 0.5 volume of air per volume of medium per minute. When the greater quantity of

carbohydrate in the all-corn mash is taken into account and the fermentation efficiency is calculated (actual alcohol yield/theoretical alcohol yield), it is found that the best mold preparations gave more complete saccharification than did

TABLE 5

*Use of culture liquors from Aspergillus niger NRRL 337 as a saccharifying agent in the alcoholic fermentation of corn*

AERATION RATE	AGE OF CULTURE	AMYLASE POTENCY	ALCOHOL YIELDS WITH VARIOUS LEVELS OF MOLD CULTURE LIQUOR (PER CENT OF MASH)*		
			Proof gallons/bushel		
<i>L air/L medium/ minute</i>	<i>days</i>	<i>units/ml</i>	<i>8 per cent level</i>	<i>13 per cent level</i>	<i>20 per cent level</i>
0.25	3	1.4		3.99	
0.25	4	1.8		4.02	
0.25	7	2.4		4.24	
0.5	2	4.8	4.06	4.52	4.86
0.5	4	7.9	4.62	5.26	5.50
0.5	7	9.0	5.00	5.37	5.40
1.0	2	6.2	4.33	5.02	5.21
1.0	4	12.9	4.97	5.10	5.24
1.0	7	22.5	5.10	5.24	5.25

\*Malt control (10 per cent of mash bill) gave 5.15 proof gallons per bushel.

TABLE 6

*Dextrinizing amylase, maltase, and total saccharifying power of various mold culture liquors\**

CULTURE	NRRL NO.	DEXTRINIZING ACTIVITY	MALTASE ACTIVITY	ALCOHOL YIELD
		<i>units/ml</i>	<i>per cent hydrolysis of maltose</i>	<i>proof gallons/ bushel</i>
<i>Aspergillus niger</i> .....	330	3.9	64.5	5.11
<i>Aspergillus niger</i> .....	679	2.7	57.4	5.25
<i>Aspergillus niger</i> .....	326	5.6	57.3	5.17
<i>Aspergillus niger</i> .....	337	11.2	39.5	5.12
<i>Aspergillus niger</i> .....	363	1.8	18.9	4.70
<i>Aspergillus oryzae</i> .....	694	13.9	17.6	4.96
<i>Rhizopus boulard</i> .....	1891	0.1	17.4	4.45
<i>Aspergillus wentii</i> .....	377	0.9	8.3	3.58
<i>Aspergillus oryzae</i> .....	449	7.7	6.2	4.37
<i>Aspergillus foetidus</i> .....	341	3.2	4.7	4.45

\* Mold culture liquors were employed in all cases at a 10 per cent level for the saccharification of grain mashes.

the barley malt. Mold preparations yielding 5.4 proof gallons per bushel of grain gave a fermentation efficiency of 86 per cent in contrast to 84 per cent for malt.

*Maltase production and its effect upon saccharification.* It may be noted from the preceding experiment that the highest yields of alcohol were obtained not

with liquors most potent in dextrinizing enzyme but rather with those of intermediate dextrinizing activity. This indicated that other enzymes capable of functioning in the hydrolysis of starch were being produced. Confirmation of this view was established by evaluating a large number of culture liquors of known dextrinizing enzyme potency for their saccharifying ability as determined by fermentation tests. The results clearly showed that saccharification of grain mashes was not well correlated with dextrinizing potency of the enzyme preparations. In view of the important role of maltase in the hydrolysis of starch (Schwimmer, 1945) the various preparations were then examined for their activity in respect to this enzyme. A summary of data on the dextrinizing potency, maltase activity, and total saccharifying power (fermentable sugar) produced by some of the cultures investigated is shown in table 6.

It is apparent that whereas dextrinizing amylase is necessary, good saccharification results with liquors relatively low in dextrinizing power if their maltase activity is high. For example, the strains *Aspergillus niger* NRRL 330, 679, and 326 gave more fermentable sugar than did *A. oryzae* NRRL 694 and *A. oryzae* NRRL 449, whereas the liquors of the last two were characterized by a much higher ratio of dextrinizing amylase to maltase. These experiments indicate, also, that the aspergilli can be evaluated as substitutes for malt by the determination of their ability to elaborate maltase and dextrinizing enzymes. However, *Rhizopus* culture liquors from NRRL 1891 contain an effective amylase complex, despite the lack of  $\alpha$ -amylase such as is found in malt and the aspergilli, and thus may contain another type of enzyme to complement the maltase present. None of the molds studied synthesized an amylase with properties comparable to the  $\beta$ -amylase of cereals.

#### DISCUSSION

That molds when cultured under submerged, aerobic conditions vary considerably in ability to produce starch-hydrolyzing enzymes has been shown in the present study. Of more than 350 cultures examined, only a limited number were capable of elaborating a dextrinizing or  $\alpha$ -amylase type enzyme, whereas a few of these produced an enzyme complex which both dextrinized and saccharified starch at a rapid rate. This occurrence among the molds of both dextrinizing and saccharifying enzymes has been reported similarly for certain amylolytic bacteria (Kneen and Beckord, 1946).

Although the dextrinizing enzyme formed by the aspergilli has properties in common with the  $\alpha$ -amylase of malt, no evidence was found to indicate that actively saccharifying mold filtrates contained enzymes comparable to the  $\beta$ -amylase of malt. Data (to be published) from a study of several organisms confirm this conclusion. In place of  $\beta$ -type amylase, the dextrinizing enzyme of saccharifying types of mold filtrates is complemented by maltase.

The efficiency of conversion of grain mashes for alcoholic fermentation, likewise, was dependent upon the maltase concentration provided the converting agent contained at least 2 to 3 dextrinizing units per ml (*A. niger* strains NRRL 326, 679, 337, and 330). Much higher concentrations of dextrinizing enzyme

with limited maltase failed to increase either the rate or the degree of saccharification of grain mashes (*A. oryzae* NRRL 694). This is of special interest in view of the indicated correlation between  $\alpha$ -amylase potency and yield of alcohol with distillers' malts (Thorne *et al.*, 1945). It must be assumed, therefore, either that malt  $\alpha$ -amylase is capable of more complete breakdown of starch than is the corresponding enzyme from mold, or that other enzyme components of malt are more active in saccharification than generally believed. Mold amylases might also comprise other enzymes than the two demonstrated to be present, as manifested by the amylolytic activity of preparations from a strain of *Rhizopus* NRRL 1891 (labeled *Rhizopus* "Boulard" as received), which display limited dextrinizing potency.

It may be of interest to compare dry weights and dextrinizing units obtained in mashes wherein good alcohol yields resulted with fungal amylases with corresponding figures for the barley malt control mash. Thus in the best alcoholic fermentation obtained with fungal amylases as presented in table 5 a culture liquor containing about 2.5 per cent of dry solids and supplied at the level of 20 per cent of the mash volume contributed about 1.25 g of solids and 395 dextrinizing units of  $\alpha$ -amylase and resulted in a yield of 5.50 proof gallons of alcohol per bushel of grain. In the barley malt control fermentation 5.0 g of malt with an  $\alpha$ -amylase activity of 24 units per g (dry basis) and a mixture content of 8.05 per cent contributed 4.6 g of dry solids and 110 dextrinizing units of  $\alpha$ -amylase and gave a yield of 5.15 proof gallons of alcohol per bushel of grain. These figures demonstrate that good preparations of fungal amylase offer higher  $\alpha$ -amylase activity per unit of dry weight than does barley malt and that the higher yields of alcohol were associated with greater dextrinizing activity of the mold preparation employed.

The media and techniques employed in the present study might well be utilized industrially for the production of fungal amylases. By selection of the proper culture, products rich in dextrinizing or both dextrinizing and saccharifying enzymes could be obtained. A large number of substrates, now by-products or waste products of industrial grain processing, could be employed satisfactorily. Culture liquors, when feasible, could be utilized without prior treatment or the enzymes could be concentrated and recovered as dry preparations as is now done with enzymes produced by *Aspergillus oryzae* cultivated on cereal bran (mold bran). Drying of the culture liquor after the removal of mold mycelium and suspended solids would result in products having amylase potency 40- to 50-fold greater than that in the untreated culture.

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#### SUMMARY

The ability of more than 350 fungi to produce amylase when grown under submerged, aerobic conditions has been determined. Cultures of *Rhizopus*



*Mucor*, *Penicillium*, *Aspergillus*, and *Monilia* were represented. With the exception of a few species of *Aspergillus*, all of the organisms investigated elaborated only limited quantities of amylase or were incapable of its formation. Among the aspergilli, substantial amounts of dextrinizing enzyme were produced by *A. wentii*, *A. oryzae*, and *A. alliaceus*, whereas both dextrinizing and saccharifying enzymes were formed by a few strains of *A. niger*. The presence of maltase in appreciable quantities was noted among the strains which actively saccharified starch.

High amylase-producing strains of *Aspergillus niger* such as NRRL 337 were readily grown on a medium composed of thin stillage supplemented with 2 per cent of corn meal and 0.5 per cent of calcium carbonate. After incubation under continuous aeration 3 to 5 days, culture liquors were satisfactory replacements for distillers' malt in the alcoholic fermentation of corn.

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# EFFECT OF THE COMPOSITION OF THE SPORULATION MEDIUM ON CITRIC ACID PRODUCTION BY *ASPERGILLUS* *NIGER* IN SUBMERGED CULTURE<sup>1</sup>

P. SHU AND M. J. JOHNSON

*Department of Biochemistry, College of Agriculture, University  
of Wisconsin, Madison, Wisconsin*

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Extensive work has been done (Foster, 1939; Perlman, Dorrell, and Johnson, 1946; Porges, 1932) on the nutritional requirements of *Aspergillus niger* and on the relation between the constitution of the fermentation medium and the yield of citric acid by this organism. However, no work has been reported in which the effect of the composition of the sporulation medium has been studied. Doelger and Prescott (1934), however, have noted that successive transfers of the culture on a synthetic medium increased citric acid yields. In the course of a study of citric acid production by submerged culture (Shu and Johnson, 1947) it was noted that the composition of the sporulation medium had a great effect on citric acid yield in fermentations in which the spores were used as inoculum. The experiments reported in the present paper were designed to determine the cause of this variation in yield.

## METHODS

A strain of *Aspergillus niger* from culture 72-4 (Perlman, Kita, and Peterson, 1946) was used throughout the experiments. The stock culture was carried on soil. In order to reduce the amount of soil substances carried over, cultures were carried through three successive sucrose agar slants made with medium A, shown in table 1. The second of these transfers was kept as a substock culture for the entire experiment. A water suspension of spores was made from the third transfer with 5 ml of sterile distilled water.

This suspension was used to inoculate the agar medium under investigation: 1 loopful for an agar slant and 0.5 ml for a bottle plate. All slants were made with 4 ml of agar medium in 18-by-150-mm pyrex test tubes. The slope of the slants was made approximately 15 degrees with respect to the axis of the tube. Bottle plates were made with 25 ml of agar medium in a 6-oz rectangular bottle. This amount gave a layer 0.5 cm thick with a 72 sq cm agar surface when the bottles were placed in a horizontal position. The media were sterilized at 120 C for 20 minutes. The inoculated slants or plates were incubated at 30 C until the entire agar surface was uniformly covered with spores.

Suspensions of spores grown on experimental agar media were made with 5 ml of sterile water for slants and with 50 ml for bottle plates. Approximately 1.5 ml

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of the suspension was used to inoculate 500-ml cotton-plugged Erlenmeyer flasks containing 50 ml of fermentation medium. The composition of the fermentation medium is shown as medium B in table 1. The inoculated flasks were incubated at 25 C on a shaker, rotating horizontally, describing a circle 1 inch in diameter at a speed of 270 rpm. All results reported represent the average of triplicate flasks.

At intervals of 5, 7, and 10 days, samples were taken and analyses were made for residual sugar, titratable acidity, and, in some cases, citric acid. The figures given in the tables are for samples taken at 10 days except when otherwise stated. Residual sugar was determined by the method of Shaffer and Somogyi (1933) and citric acid by the method of Perlman, Lardy, and Johnson (1944). The titratable

TABLE 1  
*Composition of media*

CONSTITUENTS	MEDIUM A	MEDIUM B	MEDIUM C
	<i>wt/L</i>	<i>wt/L</i>	<i>wt/L</i>
Domino sucrose	140 g	140 g	140 g
Difco agar	20 g		
KH <sub>2</sub> PO <sub>4</sub>	1 g	1 g	1 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.25 g	0.25 g	0.25 g
NH <sub>4</sub> NO <sub>3</sub>	2.5 g	2.5 g	2.5 g
HCl	to pH 4.5	to pH 3.1	to pH 2.3
Trace metals			
Cu <sup>++</sup>	0.14 mg	0.06 mg	0.06 mg
Zn <sup>++</sup>	1.4 mg	0.25 mg	0.25 mg
Fe <sup>++</sup>	2.2 mg	1.3 mg	0.45 mg
Mn <sup>++</sup>	<1 µg	<1 µg	<1 µg

The listed quantities of metals include the amounts present as impurities in other constituents of the media.

acidity was expressed in terms of anhydrous citric acid. The presence of certain trace elements was determined colorimetrically with the  $\gamma$ ,  $\gamma'$  dipyridyl method for iron; dithizone for zinc; carbamate for copper; and periodate for manganese (Sandell, 1944). The yield of citric acid was expressed as the percentage of added sugar (grams anhydrous citric acid per 100 grams added sucrose).

#### EXPERIMENTAL RESULTS

*Effect of medium on rate of sporulation.* Rectangular bottle plates were used in these experiments. The composition of the media tested was the same as medium A, table 1, except for the components which were varied. The minimum time required for the spores to cover the entire plate was used as a measure of the rate of spore formation. The results are summarized in table 2. Increasing the concentration of Zn, NH<sub>4</sub>NO<sub>3</sub>, and KH<sub>2</sub>PO<sub>4</sub> retarded the rate of spore formation, but increasing the concentration of Mn or malt extract favored spore formation. Abundant spores were formed in 48 hours if Mn or malt extract was added.

*Effect of trace metals in sporulation medium on citric acid yield.* Agar slants

were used in these experiments. Table 3 summarizes the results of the addition of Fe, Cu, Zn, and Mn, and their combinations, to the basal agar sporulation medium A. The presence of Mn at a level of 9.3 mg per liter lowered the acid yield to 35 per cent, which is only 50 per cent of the basal medium control. Iron added at a level of 8 mg per liter of medium also showed considerable effect.

TABLE 2  
*Effect of the composition of medium on rate of sporulation*

CONSTITUENT VARIED IN MEDIUM A	QUANTITY PRESENT	MINIMUM TIME FOR SPORES TO COVER AGAR SURFACE
		<i>hours</i>
None		68
pH	6.0	68
	7.5	96
Sucrose	200 g/L	68
	50 g/L	68
KH <sub>2</sub> PO <sub>4</sub>	5.0 g/L	>240
	2.5 g/L	72
NH <sub>4</sub> NO <sub>3</sub>	5.0 g/L	>240
	0.5 g/L	52
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g/L	68
	0.05 g/L	68
Mn <sup>++</sup>	9.3 mg/L	48
Fe <sup>+++</sup>	10.2 mg/L	60
	3.0 mg/L	68
Cu <sup>++</sup>	3.5 mg/L	68
	0.5 mg/L	68
Zn <sup>++</sup>	25.4 mg/L	144
	3.8 mg/L	96
Malt extract	1.5 g/L	48

Zinc alone did not exhibit any significant effect, but it exhibited some antagonistic effect against manganese. Copper showed a similar effect. Simultaneous addition of Cu, Zn, and Fe at levels of 0.34 mg, 2.4 mg, and 0.8 mg, respectively, per liter of basal medium was found to give the highest acid yield in the fermentation test. A yield of 80 per cent total acidity calculated as citric acid on added sugar was obtained in 10 days of fermentation. About 90 per cent of this total acidity was due to citric acid.

The stability of the culture in the medium (no. 18, table 3) was tested by 18 successive spore transfers. At intervals of 6 transfers, fermentation tests were

made. The results are shown in table 4. No significant changes in acid production were observed.

TABLE 3

*Effect of metallic ions in sporulation media on acid production*

NO.	METALLIC ION ADDED TO SPORULATION MEDIUM A				YIELD OF ACID* IN MEDIUM B ON AVAILABLE SUGAR PER CENT
	Mn	Zn	Cu	Fe	
	mg/L	mg/L	mg/L	mg/L	
1	0	0	0	0	70
2	93	0	0	0	14
3	9.3	0	0	0	35
4	1.9	0	0	0	53
5	0	2.4	0	0	69
6	0	2.4	0	0	71
7	0	0	3.4	0	60
8	0	0	0.34	0	66
9	0	0	0.07	0	54
10	0	0	0	8	48
11	0	0	0	0.8	53
12	0	2.4	0.34	0	63
13	9.3	2.4	0	0	45
14	0	2.4	0	0.8	70
15	9.3	0	0.34	0	45
16	9.3	0	0	0.8	37
17	0	0	0.34	0.8	56
18	0	2.4	0.34	0.8	80
19	9.3	2.4	0.34	0	46
20	9.3	2.4	0	0.8	22
21	9.3	0	0.34	0.8	20
22	9.3	2.4	0.34	0.8	54

\* Titratable acidity calculated as anhydrous citric acid.

TABLE 4

*Effect of successive spore transfers of the culture on acid production*

NUMBER OF TRANSFERS	YIELD OF ACID* ON AVAILABLE SUGAR PER CENT
0	80
6	80
12	87
18	75

\* Titratable acidity calculated as anhydrous citric acid.

The metals might exert the effects shown in table 3 either by being carried over by the spores into the fermentation medium, or by causing some physiological changes in the spores. Since it is known (Perlman, Dorrell, and Johnson, 1946) that the presence of appreciable quantities of manganese in the fermentation medium reduces yields of citric acid in surface fermentations, it seemed desirable

to determine the quantity of Mn added to the fermentation medium by the spore inoculum. Spores were grown in bottle agar plates containing various levels of Mn. Spore suspensions from each of the bottle plates were made with 50 ml sterile distilled water containing 10 per cent ethyl alcohol. The suspensions were filtered aseptically through glass wool into previously sterilized centrifuge tubes. The tubes were then centrifuged and the supernatant was pipetted out. The spores were washed twice with 50-ml portions of distilled water and finally re-suspended in 50 ml distilled water. For each of the fermentation flasks 1.5 ml of this suspension was used as inoculum. The remaining spore suspension was used for the determination of manganese.

TABLE 5

*Effect on acid production of manganese carried into fermentation medium with spore inoculum*

NO.	Mn ADDED TO SPORULATION MEDIUM A	Mn CARRIED TO FERMENTATION MEDIUM B	Mn ADDED TO FERMENTATION MEDIUM B	YIELD OF TITRA- TABLE ACID ON AVAILABLE SUGAR	YIELD OF CITRIC ACID* ON	
					Available sugar	Utilized sugar
	mg/L	µg/L	µg/L	per cent	per cent	per cent
1†	0	<0.02	0	66	57	64
2	0	<0.02	0.4	74	69	75
3	0	<0.02	3	50	45	56
4	0	<0.02	15	23	19	21
5†	0.93	0.4	0	68	57	66
6†	9.3	3	0	44	40	56
7†	93.0	16	0	21		

\* By pentabromoacetone method.

† Results of 12 days' fermentation.

Another series of fermentations was prepared and inoculated with spores produced on the basal (Mn-free) medium. To these flasks were added amounts of manganese equal to those introduced to the first series of fermentation flasks with the spores grown on the Mn-containing media. The results are summarized in table 5. It may be seen that the amount of Mn carried over with the washed spore inoculum to the fermentation medium is sufficient to retard the acid production, and that as little as 3 µg Mn per liter of fermentation medium appreciably lowers the citric acid yield.

*Addition of malt extract.* As shown in table 6, the addition of Trommer's malt extract to the sporulation medium at a level of 1.5 g per liter decreased the acid yield. The organic components of the malt extract seem to be responsible for this reduction, because the addition of the equivalent amount of the malt extract ash to the agar plate medium favored acid production in the fermentation test. Furthermore, the addition of malt extract to the agar medium containing manganese at a level of 9.3 mg per liter exhibited a definite additional influence on acid production. The results are shown in table 7. This effect of Mn and malt

extract is not noticeable if the fermentation test is run by the surface culture method (table 6) with the fermentation medium C (table 1).

TABLE 6  
*Effect of addition of malt extract (to sporulation medium) on acid production*

SUBSTANCES ADDED	QUANTITY ADDED	YIELD OF ACID PRESENT*	METHOD OF FERMENTATION
	g/L		
None.....		66	submerged
Trommer malt extract.....	1.5	55	submerged
Trommer malt extract ash.....	Equivalent to 1.5 g malt extract	76	submerged
Trommer malt extract.....	1.5	28	submerged
Mn.....	0.0093		
None.....		60	surface
Trommer malt extract.....	1.5	57	surface
Mn.....	0.0093		

\* Titratable acidity calculated as anhydrous citric acid.

TABLE 7  
*Retardation of acid production by simultaneous presence of malt extract and manganese in sporulation medium*

BASAL MEDIUM A +		YIELD OF ACID ON AVAILABLE SUGAR*
Mn	Trommer malt extract	
mg/L	g/L	per cent
9.3	0.00	33
9.3	0.01	27
9.3	0.05	12
9.3	0.10	10
9.3	1.00	11

\* Titratable acid calculated as anhydrous citric acid on 7 days' fermentation.

#### SUMMARY

The addition of Mn and Trommer malt extract at a level of 9.3 mg and 1.5 g, respectively, to 1 liter of basal agar plate medium accelerated spore formation, whereas increasing the concentration of  $\text{KH}_2\text{PO}_4$ ,  $\text{NH}_4\text{NO}_3$ , and Zn retarded spore formation.

The presence of Mn in the sporulation medium at a level of 9.3 mg per liter retarded citric acid production in submerged fermentations in which the spores were used as inoculum. The effect is shown to be attributable to the amounts of Mn carried over into the fermentation medium by the washed spore inoculum.

The presence of Mn and malt extract in the sporulation medium reduced the acid production in submerged fermentation, but not in the surface culture fermentation.



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# CLOSTRIDIA IN GAS GANGRENE AND LOCAL ANAEROBIC INFECTIONS DURING THE ITALIAN CAMPAIGN

AARON H. STOCK<sup>1,2</sup>

*2nd Medical Laboratory, United States Army*

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When World War II began, some of the experimental results obtained on animals indicated that sulfonamides applied locally and taken by mouth might prevent infections with anaerobes of the gas gangrene group. It became evident, even in the Western Desert and Tunisia, that anaerobic infections would occur in spite of sulfonamide prophylaxis. When the fighting took place on the more cultivated soil in Sicily and Italy, the incidence of gas gangrene increased. Antiserums made in the United States, usually containing antitoxin only for *Clostridium perfringens* (*B. welchii*) and *Clostridium septicum*, did not appear to prevent gas gangrene and were of limited value in the therapy of cases (Jergesen, 1944). The question arose as to the incidence of *Clostridium novyi* (*B. oedematiens*) in anaerobic infections; it was questioned if the poor results with serum could be attributed to the lack of *C. novyi* antitoxin in some American polyvalent gas gangrene antiserums.

In order to determine the incidence of *C. novyi*, the clostridial flora of 25 cases of gas gangrene that occurred in Italy was studied (Stock, 1944). In a second study, made while the fighting was in the Northern Apennines, 5 additional cases of gas gangrene and 7 of local anaerobic infections were cultured, and at the same time an effort was made to determine the incidence and significance of positive blood cultures. It appeared important to learn whether therapy could save a case of gas gangrene once the causative organisms had entered the blood stream. Although only the preliminary phase of the latter study was completed, it may be of value to record these results and to summarize our entire findings because so few reports on cultures in gas gangrene or other anaerobic infection in World War II have appeared (MacLennan, 1943, 1944; MacLennan and Macfarlane, 1944; Jeffrey and Thomson, 1944; Smith and George, 1946).

## MATERIALS AND METHODS

Specimens of muscle or blood were placed in chopped meat medium at surgical operation and forwarded to the laboratory. Anaerobic jars of the McIntosh-Fildes type, but without a heating coil, were fashioned from 105-mm shell cases (see Smith and George, 1946). Two g of palladium-asbestos (Fildes, 1917) covered by a wire screen served as catalyst. Anaerobes were grown on the surface of thioglycolate blood agar plates from inoculums of unheated, heated (80 C), and enriched heated samples. Isolation and identification of clostridia were

<sup>1</sup> Major, Med. Corps.

<sup>2</sup> Present address: Department of Bacteriology and Immunology, School of Medicine, University of Pittsburgh, and Children's Hospital of Pittsburgh.

TABLE 1  
*Clostridia isolated from wound and blood cultures in gas gangrene and  
 local anaerobic infections*  
 (Second Series)

CASE NO.	TIME OF BLOOD CULTURE	SOURCE OF CLOSTRIDIA	CLOSTRIDIA FOUND									REMARKS
			<i>perfringens</i>	<i>novyi</i>	<i>bifermentans</i>	<i>septicum</i>	<i>tetani</i>	<i>sporogenes</i>	<i>cochlearium</i>	<i>undetermined</i>	<i>terium</i>	
Group I "Gas Gangrene"												
1	Post mortem	M* B*		+						++ ++		Died
2	Post mortem	M B	+	++			+					Died
3	Ante mortem	M B	+		+					+	+	Died
4	At operation	M	+	+								Lived
5	At operation	M	+		+			+				Lived
Group II "Local Infections"												
1	At operation	M	+	+				+	+	++		Lived
2	At operation	M	+	+	+							Lived
3	At operation§	M	+	+	+			+				Lived
4	At operation	M	+									Lived
5	At operation	M	+	+								Lived
6	At operation	M	+									Lived
7	At operation	M	+	+								Lived

\*M—Muscle culture. B—Blood culture. Only positive blood cultures are tabulated. All others were negative. (See case no. 3, local infections.)

† Surface growth like *C. septicum*. Fermented no sugars. Not proteolytic. Hemolytic. Not pathogenic for guinea pigs.

‡ Not isolated in pure culture. Overgrown by *C. tetani*.

§ Blood culture positive but technique questionable.

|| "Unidentified" strains differed in some respects from anaerobes described in literature.

accomplished by methods similar to those outlined by the Committee of London Sector Pathologists (1943) and by Reed and Orr (1941).<sup>3</sup> The cultures when isolated were replated as often as necessary for purity. For fermentations, sugar

<sup>3</sup> Some of the methods employed were suggested by Major J. D. MacLennan, R.A.M.C.

broths of lactose, glucose, sucrose, and salicin sufficed; sodium thioglycolate (BBL) and semisolid agar mediums were employed so that growth in an anaerobic jar was unnecessary (Reed and Orr, 1941). Proteolysis was determined from growth in chopped meat medium and reduced-iron milk medium. In contrast to the report of Reed and Orr (1941), strains of *C. novyi* were not proteolytic in our tests. In pathogenicity tests made by injecting guinea pigs with 18-hour cultures in thioglycolate broth, no calcium chloride was used. A few protection tests were made with monovalent antisera obtained from Major G. H. Eagles, R.A.M.C.

## RESULTS

The distribution of anaerobes in muscle and blood cultures in the two groups of cases studied in the present series is shown in table 1.

Regardless of clinical diagnosis, all wound specimens on culture showed

TABLE 2  
*Clostridia found in gas gangrene and local anaerobic infections, Italy*  
(Summary of first and second series)

	NO. OF CASES	CLOSTRIDIA										
		<i>perfringens</i>	<i>novyi</i>	<i>septicum</i>	<i>fallax</i>	<i>bifermentans</i>	<i>tetani</i>	<i>sporogenes</i>	<i>unidentified</i>	<i>cochlearium</i>	<i>terium</i>	<i>butyricum</i>
Gas gangrene.....	30	24	15	1	1	7	3	19	4	1	4	2
Local anaerobic infection...	7	7	5			2		2	1	1		

pathogenic species of clostridia. Table 2 summarizes the combined cultural results in our first series (Stock, 1944) and second series. In the cases of gas gangrene, *C. perfringens* and *C. novyi* were the most prevalent of the pathogenic clostridia. *C. septicum* was found only once.

In two of the three fatal cases of gas gangrene, blood cultures taken post mortem revealed pathogenic species of clostridia (see table 1). Only one of the other cases had a positive culture for pathogenic species of clostridia, but this finding was discounted because the blood culture bottle apparently had been contaminated with the tissue culture (case no. 3 of "local infections").

In pathogenicity tests, some strains of *C. novyi* were either nonpathogenic or of low pathogenicity. Pathogenic strains were neutralized by specific antitoxin. The strain of *C. septicum* was highly virulent. All strains of *Clostridium bifermentans* were tested but no strain produced more than tumefaction in guinea pigs. The three strains of *Clostridium tetani* were pathogenic. All strains of *C. perfringens* that were tested were virulent. The strain of *Clostridium fallax* was nonpathogenic for a guinea pig. This organism, which had the characteristics of a hemolytic *Clostridium multifementans*, has been discussed elsewhere (Stock, 1944). It should be noted that in all these tests whole broth

cultures were injected and no calcium chloride was used (Bullock and Cramer, 1919).

#### DISCUSSION

In general, the distribution of clostridia found in cases of gas gangrene was similar to that described in published reports (Weinberg and Seguin, 1918; Medical Research Committee, Brit., 1919; Sordelli, 1923; Zeissler and Neller, 1928; MacLennan, 1943, 1944; MacLennan and Macfarlane, 1944; Smith and George, 1946). The presence of *C. novyi* in about 50 per cent of cases of gas gangrene is confirmed. *C. novyi* was found in soil by Zeissler and Rassfeld (1928) in 64 per cent of samples, so that a high incidence is possible in wounds. If the prophylactic and therapeutic efficiency of gas gangrene antisera is to be determined, it would seem necessary to include *C. novyi* antitoxin in the polyvalent serum (see Hall, 1946). Gas gangrene toxoids for immunization should contain *C. novyi* toxoid as a component (Robertson and Keppie, 1943).

*C. septicum* was isolated only once in 30 cases of gas gangrene. In a large series of cases, Weinberg and Seguin (1918) found a 13 per cent and MacLennan (1943) a 19 per cent incidence for this species. On the other hand, Zeissler and Neller (1928) isolated only 1 strain of *C. septicum* from 22 cases of gas gangrene in German civilians, and Zeissler and Rassfeld (1928), in an examination of soil, found an incidence of 8 per cent.

None of the strains of *C. bifermentans* isolated in our studies was pathogenic for guinea pigs by the methods employed. It is to be noted that Clark and Hall (1937) and Stewart (1938) have found *C. bifermentans* antiserum of protective value against the pathogenic variety of this species (*Clostridium sordellii*).

No strains could be identified culturally or by pathogenicity tests as *Clostridium histolyticum*. In soil, Zeissler and Rassfeld (1928) reported an incidence of 2 per cent for this species. Smith and George (1946) working in Italy did not find strains of *C. histolyticum*. In their series, Weinberg and Seguin (1918) isolated 8 strains from cases late in the investigation. In MacLennan's series (1943), all 9 patients with *C. histolyticum* in the wound flora succumbed.

Death from gas gangrene has been attributed generally to toxemia (MacLennan, 1946). Bacteremia which is known to occur has been considered a terminal event, although this conclusion, drawn from Weinberg and Seguin's paper (1918), may not be warranted. Further studies on blood cultures in gas gangrene are needed to determine whether bacteremia is an additional factor in the high mortality rate which still exists in spite of present therapeutic agents and surgical technique. There is experimental evidence that is suggestive, for once clostridia had entered the blood stream in infected mice, which occurred after 3 hours, McIntosh and Selbie (1943*a*, 1943*b*) found local chemotherapy to be less effective.

In 7 local anaerobic infections cultured in Italy, 7 strains of *C. perfringens* and 5 of *C. novyi* were found. Thus, pathogenic species of clostridia were commonly found and were not less frequent than proteolytic nonpathogenic clostridia. This is the opposite of the findings in a small series of cases of "anaerobic

cellulitis" cultured by MacLennan (1943) in the Western Desert, but agrees with those of Weinberg and Seguin (1918) in the cases called "gaseous phlegmon" or "gaseous wounds." In our experience, "heavy local anaerobic infection" used by Robertson (1929) may be a more accurate description of the lesions seen in Italy than "anaerobic cellulitis." Beginning with local anaerobic infections in dead tissue, all gradations and degrees of infection resulted, with fulminating gas gangrene at the extreme. Debridement removed infected tissue and often prevented further spread. Prophylactic penicillin was used in wounded patients in the latter part of the Italian campaign, but no data are available on its effect on the cultural findings of the bacterial flora of the wounds or on its therapeutic value in the dosage used.

Numerous nonpathogenic species of clostridia and many aerobes (not listed in our tables), particularly nonhemolytic streptococci, were found in the specimens from gas gangrene and local anaerobic infections but were not investigated further. No information was obtained on their significance.

#### ACKNOWLEDGMENT

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#### SUMMARY

In 30 cases of gas gangrene cultured in Italy, 80 per cent of the cases showed *Clostridium perfringens*, 50 per cent *Clostridium novyi*, and 1 case *Clostridium septicum*. The high incidence of *Clostridium novyi* confirms the earlier reports of French and British investigators. *Clostridium tetani* and nonpathogenic *Clostridium bifermentans* were found. No strains of *Clostridium histolyticum* were identified. In a trial series, clostridia were recovered post mortem from blood cultures in 2 cases of gas gangrene. No data were obtained on the prognostic significance of a positive blood culture. Seven local anaerobic infections showed on culture 7 *Clostridium perfringens* and 5 *Clostridium novyi*.

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## ROUGH-SMOOTH DISSOCIATION OF *NEISSERIA* *INTRACELLULARIS*

FLORENCE L. EVANS

*Department of Pathology and Bacteriology, Louisiana State University School of Medicine,  
New Orleans, Louisiana*

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Dissociation in the *Neisseria* group is infrequently reported, therefore the following occurrence may be worthy of note.

The culture was a stock strain of *Neisseria intracellularis* Gordon type III which had been maintained in stock by the author for about 2 years prior to the appearance of the variant. It had been cultivated for about a year on human blood agar slants and subsequently on Dorset's egg medium with frequent platings on blood agar.

The variation was first noted on a plate of human blood agar containing 0.5 per cent glucose after 24 hours' incubation at 37 C and 24 hours at about 29 C. The variation appeared to be of the simple R-S type. About 50 per cent of the colonies were typical meningococcus colonies and about 50 per cent were of the variant type. The R or variant colonies were about half the size of most of the typical S colonies. The surface of the R colonies was warty and the margins were irregular. These colonies had a heaped-up appearance and were pinkish or yellowish pink in color, in marked contrast to the typical or smooth colony. When the R colony was picked off the medium, the entire structure came away intact. It was found to be extremely hard and could only be broken up by being ground between two glass slides.

Transfers of the two colony types to blood agar plates of the same composition gave the following results: The typical S type colonies gave rise to pure cultures of S colonies through successive subcultures. The R or variant type gave cultures which consisted of about half typical colonies and half R colonies for five successive transplants. The smooth colonies from these plates invariably gave rise to pure cultures of typical colonies.

Transfers to plates of 10 per cent ascitic fluid agar containing 0.5 per cent glucose resulted in 100 per cent typical smooth *Neisseria intracellularis* colonies with either type as an inoculum. Transfers to Avery's blood broth with subsequent streaking on the ascitic fluid agar also resulted in pure cultures of the S type of colony. Laked blood agar prepared from the same blood used for the blood agar plates resulted in half R and half S colonies, provided they were inoculated with material from an R colony.

A sudden change occurred in transplants from the plates of the fifth successive passage of the R type of colony. The sixth successive passage on blood agar plates and laked blood agar plates gave a pure culture of a colony which resembled the original R, except in size. This new colony was much smaller than the original variant. At 24 hours it was microscopic in size, and at maximum develop-

ment (3 days) was about 1 mm in diameter. When these small R colonies were transferred to blood agar plates, they gave rise to pure cultures of similar colonies. When transferred to ascitic agar plates they maintained their characteristics. Growth in Avery's blood broth which was streaked on ascitic agar also gave pure culture of the small R type. This small R type of colony was carried for 10 successive generations on ascitic agar, blood agar, and Avery's blood broth without any indication of further change in colony morphology or any tendency to revert to the normal type of colony.

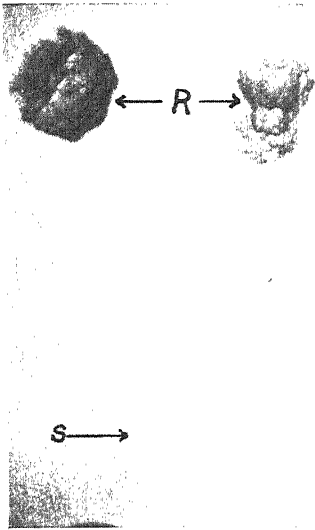


FIG. 1

FIG. 1. ROUGH-SMOOTH DISSOCIATION. BLOOD AGAR PLATE, REFLECTED LIGHT.  
MAGNIFICATION, APPROXIMATELY 10 DIAMETERS

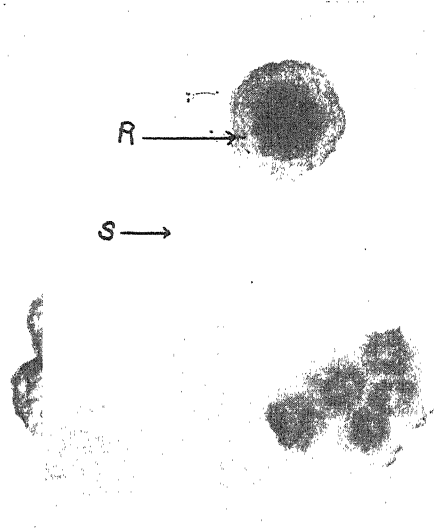


FIG. 2

FIG. 2. ROUGH-SMOOTH DISSOCIATION. BLOOD AGAR PLATE, TRANSMITTED LIGHT.  
MAGNIFICATION, APPROXIMATELY 10 DIAMETERS

Microscopic examination of the various types of colonies showed them to consist entirely of gram-negative cocci and diplococci morphologically resembling *Neisseria*. Fermentation studies on the large R type could not be run because the cultures invariably reverted to the S type in the fermentation tubes. Such tests, however, showed the production of acid from glucose and maltose. The small R type, which was stable, produced a faint acidity in glucose, but none in maltose, sucrose, lactose, dextrin, inulin, or xylose.

The production of this variant was apparently due to the accidental use of human blood which contained antimeningococcus antibodies. This is substantiated by the following observations: First, the S or typical colony produced wide dense halos, sometimes one-half to three-fourths inches in diameter on plates prepared with this specimen of blood. Secondly, 2 months after the original observation, material from the same tube that had produced the dissociated colonies was plated on media of identical composition, except that the blood was

from a different donor. It yielded a pure culture of typical S type *Neisseria intracellularis* and no R colonies could be found on numerous plates. Thirdly, plasma obtained by centrifuging some of the same blood that produced the dissociation gave the following results in the agglutination tests: When the antigen was the Gordon type III strain, grown on ascitic agar, agglutination was positive in a dilution of 1:320. A satisfactory suspension of the small R colonies could not be prepared, and therefore the test was not satisfactory with this antigen. Similar antigens of other strains of *Neisseria intracellularis* all gave negative agglutination with this plasma. These strains included the other Gordon types and several strains isolated locally from cases of meningitis.

#### SUMMARY

A stable R variant of Gordon type III *Neisseria intracellularis* that has lost the ability to ferment maltose and that was apparently induced by antibodies is reported.



# DESCRIPTION OF STRAIN C27: A MOTILE ORGANISM WITH THE MAJOR ANTIGEN OF SHIGELLA SONNEI PHASE I

W. W. FERGUSON AND N. D. HENDERSON

*Bureau of Laboratories, Michigan Department of Health, Lansing, Michigan*

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The development of typing sera for antigenic analysis of *Salmonella* organisms has made possible the discovery of *Salmonella* antigens in cultures of other genera. Reports of such antigenic interrelationships have become common in the literature. Similarly, the development of typing sera for the detection of type-specific antigens in *Shigella* is making possible the discovery of antigenic relationships between *Shigella* and organisms of other genera.

In a paper devoted to the typing of *Shigella*, Ferguson *et al.* (1947) referred to a motile organism of the family *Enterobacteriaceae* which bears the major antigen of *Shigella sonnei* phase I, as defined by Wheeler and Mickle (1945). A detailed description of this organism, which has been given the strain designation C27, is contained in the present report.

Isolation of the culture was effected on MacConkey agar plates from a fecal specimen submitted to this laboratory for culture. No clinical history was indicated on the specimen blank, and efforts to obtain a history from the physician have been unsuccessful.

The identification of culture C27 was attempted by the methods used routinely in our diagnostic laboratories. Lactose-nonfermenting colonies were transferred from a MacConkey plate to triple sugar agar slants on which growth produced a *Shigella*-like reaction. Upon test with highly absorbed *Shigella* sera representative of the types commonly encountered in Michigan, the growth from triple sugar slants reacted strongly in *Shigella sonnei* phase I antiserum. No reaction occurred in any of the other sera. Biochemical and other tests revealed, however, that C27 differed in several respects from *Shigella sonnei*.

Strain C27 was found to be a gram-negative, nonsporulating rod form, actively motile when grown at room or incubator temperatures. Motility was sufficiently pronounced that growth spread through a 6-inch column of semisolid agar in 12 hours. Flagellar stain preparations, made after the method of Leifson (1938) with Baltimore Biological Laboratory stain, revealed that C27 was apparently amphitrichous.

The organism grew readily at 37 C on nutrient agar, on SS, and MacConkey media, and on veal infusion blood agar. On the latter medium, small but clear zones of hemolysis were produced around the massed growth or isolated colonies. On blood agar the colonies appeared gray, shiny, and opaque, with slightly raised centers, smooth surfaces, and entire edges. Colony size varied from 2 to 4 mm.

Strain C27 was an anaerogenic culture which fermented glucose and maltose in 24 hours and produced acid from salicin in 7 days. Sucrose, mannitol,

sorbitol, dulcitol, rhamnose, and xylose were not attacked. Acetylmethylcarbinol was not formed, and urea and citrate were not utilized. Indole and  $H_2S$  were produced and trimethylamine oxide was reduced. The time of lactose fermentation was variable. On first isolation, one subculture fermented lactose after 72 hours, whereas a variant fermented the carbohydrate in 48 hours. The latter, after serial transfer, fermented lactose in 24 hours.

Immune sera for strain C27 were developed in two rabbits whose sera prior to immunization contained no perceptible agglutinins for C27 or *Shigella sonnei* phase I organisms. *Shigella sonnei* phase I antiserum unabsorbed and *S. sonnei* absorbed serum freed of cross reactions as described by Ferguson, Branston, MacCallum, and Carlson (1947) were available for agglutination studies. The

TABLE 1  
*Serological results*

ORGANISM	SERUM					
	<i>S. sonnei</i> , phase I			C27		
	Unabsorbed	Absorbed by		Unabsorbed	Absorbed by	
		<i>Shigella</i> sp.*	C27		<i>S. sonnei</i>	C27 boiled
<i>S. sonnei</i> , phase I living.....	10,240	10,240	<80	20,480	80	<80
<i>S. sonnei</i> , phase I alcohol-treated..	2,560	—	—	5,120	<80	—
C27, living.....	2,560	2,560	<80	20,480	5,120†	1,280†
C27, alcohol-treated.....	2,560	—	—	5,120	<80	—

Agglutinations conducted at 50 C with overnight incubation; lowest dilution 1:80;—not made.

\* *Shigella* cross reactions removed.

† Soft, floccular clumps which shake out readily.

results of such studies with absorbed and unabsorbed sera and the respective antigens are recorded in table 1.

It is obvious from a study of the table that C27 strain has a somatic antigen similar to the major antigen of *Shigella sonnei* phase I. This is borne out by the fact that C27 organisms were able to exhaust the specific agglutinins from an absorbed *Shigella sonnei* typing serum. This is also borne out by further data in the table which show that *Shigella sonnei* phase I organisms are capable of exhausting the somatic agglutinins from C27 antiserum.

Organism C27 possesses additional antigens not shared by *Shigella sonnei* which are probably contained in the flagella. It will be seen in the table that absorption of C27 antiserum with *Shigella sonnei* organisms removed agglutinins for *S. sonnei*, while a considerable residual antibody content remained for C27 untreated culture. The clumps formed by agglutination of both agar- and broth-grown suspensions of C27 with the residual agglutinins were soft and floccular—very much like the clumps formed by agglutination of *Salmonella* organisms with pure "H" antisera. Moreover, a suspension of C27 after treat-

ment with absolute alcohol after the method of Edwards and Bruner (1942) would not react with this same absorbed serum. Further evidence that a labile antigen not shared by *Shigella sonnei* is present in the C27 culture was demonstrated by absorption of C27 serum by a boiled suspension of the homologous organism. Although agglutinins for *Shigella sonnei* were removed by this treatment, residual agglutinins were left for the C27 culture.

Our conclusion that the C27 strain is related to *Shigella sonnei* phase I rather than phase II is based on examination of C27 organisms with phase I and phase II sera furnished by Dr. K. M. Wheeler, and on the outcome of tests with sera produced by us. A culture of C27 examined by Dr. Wheeler was found to react with his phase I absorbed serum. The culture of *Shigella sonnei* used by us for the production of sera and for this study is remarkable for its stability in phase I. It was carefully checked for phase throughout the study.

Since the finding of the C27 culture, a second motile, paracolonlike organism related to *Shigella sonnei* phase I has been discovered by Wheeler (personal communication). It appears possible that similar cultures may be found as the use of absorbed *Shigella* typing serum becomes widespread.

#### SUMMARY

A motile organism of the family *Enterobacteriaceae* containing a somatic antigen similar to the major antigen of *Shigella sonnei* phase I is described. Biochemically this organism appears to be an anaerogenic paracolon.

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# MORPHOLOGY OF ESCHERICHIA COLI EXPOSED TO PENICILLIN AS OBSERVED WITH THE ELECTRON MICROSCOPE

A. J. SHANAHAN, A. EISENSTARK, AND F. W. TANNER

*Department of Bacteriology, University of Illinois, Urbana, Illinois*

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The morphological effects of penicillin on gram-negative nonsporeforming rods, especially *Escherichia coli*, have been studied by several authors. Gardner (1940) reported that grotesque forms of *E. coli* resulted from autolytic swelling when this organism was treated with penicillin and that elongated, swollen cells resulted from incomplete fission. Weiss (1943) showed by electron micrographs that certain bacterial cells, when medicated with penicillin, became enlarged and fission was often incomplete. The effects of penicillin on intestinal bacteria as reported by Thomas and Levine (1945) included long twisting filaments in lower inhibitory concentrations and cells resembling Pasteur flasks, swelled fusiform rods, large globular cells, and irregular masses in higher concentrations. In concentrations just above those with visible growth, globular masses were found upon centrifugation and examination of the sediment. Normal rods were cultivated from the masses in the sediment. In the same year Altire-Werber, Lipschitz, Kashdan, and Rosenblatt (1945) studied the effect of incompletely inhibitory concentrations of penicillin on *Escherichia coli*. These authors found organisms resembling budding fungi in the urine of patients treated with penicillin. Culturing for molds was negative, but *E. coli* was isolated on other media and it was concluded that penicillin was responsible for the funguslike appearance of the cells in urine. This assumption was confirmed by *in vitro* experiments using MacConkey's agar to which was added varying concentrations of penicillin. Morphological changes noted were diphtheroidlike, bipolar cells at 75 units per ml.; unsegmented filaments with myceliallike appearance at 100 units per ml; and at 150 units per ml forms similar to those observed in the urine specimens, designated as zygosporerlike bodies. Kojima and Heimbrock (1946) and Fennel (1946) confirmed the findings of Altire-Werber *et al.* (1945). Both reports indicated that the urine of penicillin-treated patients contained budding funguslike forms, which in one case were identified as *B. aerogenes* (*Aerobacter aerogenes*) and in the other as *E. coli*. Kojima and Heimbrock did not obtain bulbous forms in broth cultures with penicillin, but Fennel found various bizarre types in glucose broth containing various concentrations of penicillin. In all of the foregoing cases, when the organisms showing atypical forms were cultured on media not containing penicillin, only normal rods were found. A short but informative review of the action of penicillin and its effect on bacterial morphology was given by Fisher (1946).

Morphological variation may be induced by agents or conditions other than by the use of penicillin. Only a few of the more important aspects of the phe-

nomena of variation need be mentioned here. Scales (1921) found several morphological types of *E. coli* including coccoidal types resembling those reported in this paper, but induced by 6 per cent sodium chloride. The influence of H ion concentration on the structure of *Hemophilus influenzae* was noted by Reed and Orr (1923). Long filaments, coccuslike forms, and a variety of swollen and elongated cells were found at pH 6.5 to the maximum acid pH allowing growth, and from pH 8.0 to the maximum alkaline pH allowing growth. In his series of studies on microbic heredity, Mellon (1925*a*, 1925*b*, 1926), observed a funguslike organism in the urine of patients treated with utropin and sodium acid phosphate. The organism was found to be *E. coli* on ordinary media, but "zygospore" formation was noted on media with inducing substances added. Coccoid forms followed by coarse filaments and rods arose from the "zygospores." The pleomorphism of *B. paratyphi* B (*Salmonella schottmuelleri*) as reported by Kritschewski and Ponomarewa (1934) is especially noteworthy because they apparently used no inducing substances, having cultured the organisms on 1 to 2 per cent raffinose agar. Their photographs give excellent evidence of the variations of form of bacterial cells. Wahlin and Almaden (1939) covered the so-called "megalmorphic phase" of bacteria in detail which the interested reader may find informative. No attempt is made here to review all of the works of Dienes and Klieneberger, but it is evident from their work that bacterial variation under normal conditions can be demonstrated by proper techniques. The appearance of fusiform bodies in colon bacillus colonies (Dienes, 1939*a*), in *L* organisms of Klieneberger and *Streptobacillus moniliformis* (Dienes, 1939*b*), in a *Flavobacterium* (Dienes, 1942), in a parainfluenza bacillus (Dienes, 1944), and in *Proteus* cultures (Dienes, 1946) is of interest due to the resemblance between these forms and those induced by penicillin in the organisms reported here and by others. The association of pleuropneumonia-like organisms with *Streptobacillus moniliformis* as reported by Klieneberger (1942) and the pleomorphism of *Bacteroides* strains as shown by Dienes and Smith (1944) are also pertinent.

#### MATERIALS AND METHODS

The methods used to demonstrate the effects of subinhibitory concentrations on *E. coli* were virtually the same as those of Alture-Werber *et al.* (1945). MacConkey's agar (Difco) was sterilized in 10-ml amounts in tubes and just before the plates were poured appropriate amounts of penicillin<sup>1</sup> were added to give final concentrations ranging from 50 to 200 units per ml. *E. coli* strain 252, University of Illinois stock culture collection, was employed as the test strain, 0.1 ml of a 1:100 dilution of a 24-hour-old culture being added to each tube. The plates were incubated at 37 C for 18 to 24 hours and gram stains made from isolated colonies obtained at the various concentrations of penicillin. Suitable colonies were selected and a suspension was made for use in preparing mounts for the electron microscope. The mounts were prepared in the usual manner using a collodion membrane, and examination of the specimens was made with the type B, RCA electron microscope. In order to demonstrate similar-

<sup>1</sup> Na-Penicillin used was supplied by the Schenley Laboratories, Lawrenceburg, Indiana.

ties and differences between light and electron microscopy of the same type of cells, strain 252 was grown on MacConkey's agar as follows: About 0.1 ml of melted MacConkey's agar containing a suitable concentration of penicillin was pipetted onto a sterile glass slide and immediately covered with a sterile cover slip. The latter was sealed with melted paraffin and the slide culture then incubated either on a warm stage at 37 C or placed in sterile petri plates at 37 C. Frequent examinations were made at intervals until the desired forms could be observed and photographed under oil immersion using a Leitz-Wetzler "makam" attached to a Leitz-Wetzler microscope; Wratten M plates were used as negatives.

#### RESULTS

*E. coli*, strain 252, was found to grow abundantly on MacConkey's agar plates in concentrations up to 100 units per ml and to a lesser degree in concentrations up to 200 units. In the lower concentrations there was little change in the morphology, but as the unitage increased more elongated and swollen cells appeared, many of them remaining only partially divided. The appearance of very large fusiform bodies was especially noted at 150 and 200 units per ml on the agar plates. They were also readily found in the slide cultures at 50 units per ml; but at this concentration there was also an abundance of normal rods, whereas with the higher concentrations nearly all of the cells were elongated and had fusiform swellings.

Several preparations were made from the agar plates and examined with the electron microscope. It was soon found that low magnification (3,000 X) gave the best results because the fusiform bodies and elongated cells were so large. The photographs shown were made from a specimen taken from MacConkey's agar plate containing 150 units per ml after incubation at 37 C for 18 hours.

Figure 1 (no. A) shows the appearance of a young fusiform body near the lower right-hand corner. The entire cell is dense and apparently homogeneous, whereas the older cells as shown toward the upper right-hand corner are entirely granulated. Elongated and partially divided dense cells may also be seen. The granular appearance surrounding the cells may or may not be of significance and this has not been determined at this time. Figure 1 (no. B) shows another type of cell commonly encountered and reveals the intense granulation which can be observed in less degree with the light microscope. Numbers C and D (figure 1) represent a type of granule found throughout the entire specimen, but again the significance of these bodies is not yet clear. Forms similar to these may be found in the slide cultures not only of strain 252 but of several others studied. Figure 1 (no. E) shows two cells only somewhat larger than normal cells, but the failure of the cells to separate is clearly seen plus the granulation common to older cells. Figure 2 (no. A) again shows partially divided cells, probably of normal size, and close examination reveals the presence of numerous flagella. It might be pointed out that this strain of *E. coli* is actively motile and that the elongated and fusiform cells are apparently likewise motile. Figure 2 (no. B)

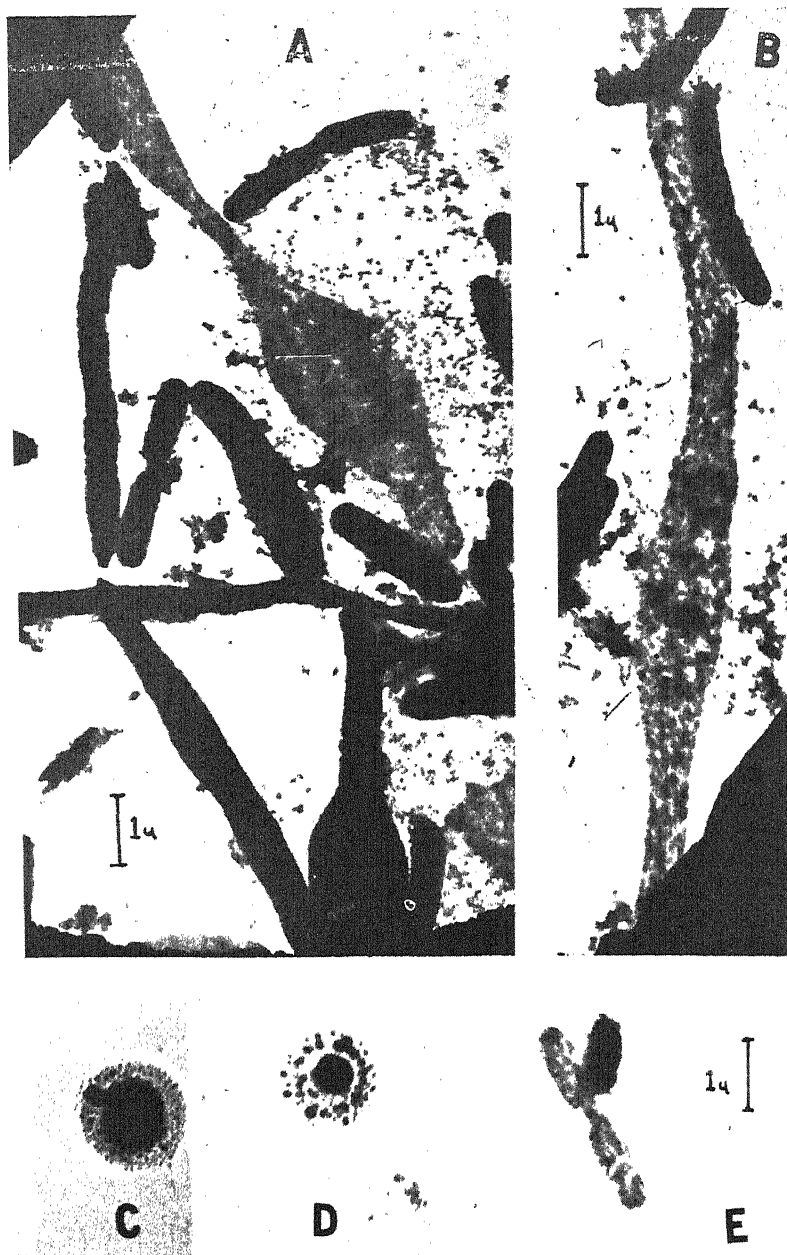


FIG. 1

## EXPLANATION OF FIGURES

The photographs in figures 1 and 2 and no. A figure 3 were all taken at a magnification of  $3,000\times$  on the electron microscope and enlarged  $4\times$  photographically. Number B figure 3 was taken of living cells under oil immersion ( $97\times$ ) with a  $10\times$  ocular and presented as a contact print, magnification approximately  $970\times$ .



FIG. 2

represents a typical field encountered with this specimen. The presence of artifacts and extraneous material could not be avoided. Figure 3 (no. A) repre-

sents another type of form frequently found, being considerably elongated and filamentous with one or several swellings along the cell although only one swollen portion is shown. Figure 3 (no. B) is included to show the appearance of this same strain of *E. coli* when grown on MacConkey's agar slide cultures with 50

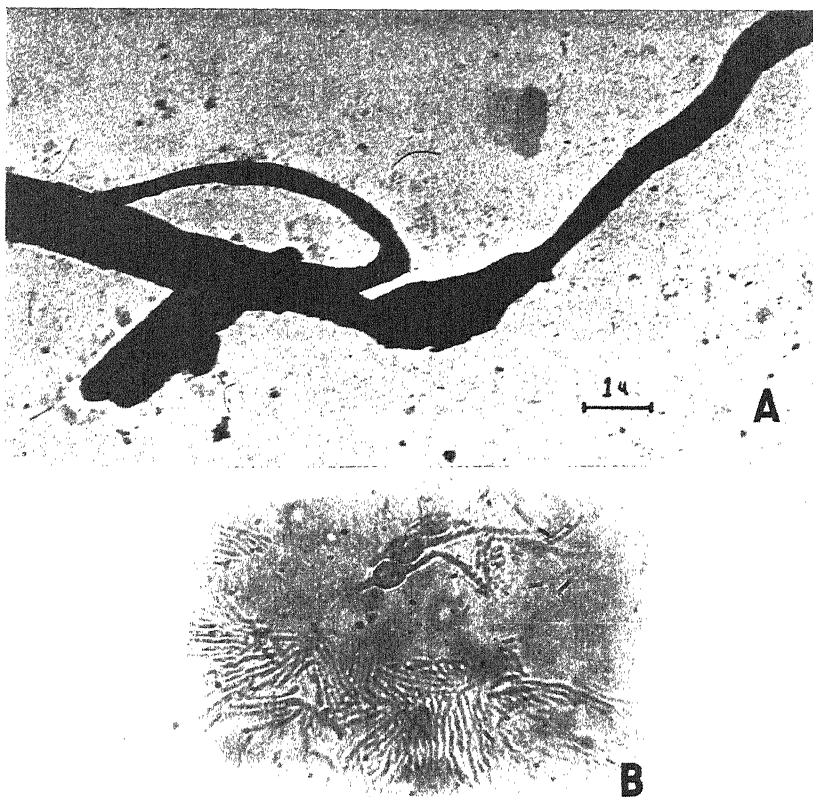


FIG. 3

units per ml of penicillin. The intense granulation of the 18-hour-old cells is quite evident, although not so well revealed as by the higher magnification obtained with the electron microscope.

#### DISCUSSION AND SUMMARY

Several electron microscope pictures are presented, as well as one light microscope photograph, revealing the form and inner structure of some of the types of cells induced by exposure to penicillin, using a strain of *Escherichia coli*. Observations under oil immersion with light microscopy indicate that the fusiform bodies arise by direct swelling of a portion of an elongated rod. No attempt is made at the present time to interpret the significance of the intense

granulation of the fusiform and rod forms as revealed by electron microscopy nor to account for the fate of these cells. Work is being continued along these lines and will be reported at a later date.

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# ACETIC ACID PRODUCTION FROM ETHANOL BY FLUORESCENT PSEUDOMONADS

R. Y. STANIER

*Department of Bacteriology, Indiana University, Bloomington, Indiana*

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Apart from the observation by Alsberg (1911) that gluconic acid is produced from glucose by *Phytomonas savastanoi*, the oxidative metabolism of fluorescent pseudomonads has received little attention until very recently. For the most part it has been tacitly assumed that these organisms, by virtue of their obligately aerobic nature, carry out a complete mineralization of organic substrates (den Dooren de Jong, 1926). However, the researches of Pervozvanski (1939*a*, 1939*b*), followed by those of Lockwood *et al.* (1941, 1946), have established the unexpected fact that the dissimilation of monosaccharides by the majority of fluorescent pseudomonads is accompanied by the production and accumulation of the corresponding hexonic or pentonic acids in large amounts. Some strains when acting on glucose also carry the oxidation further to 2-ketogluconic acid (Pervozvanski 1939*a*, Lockwood *et al.* 1941) and  $\alpha$ -ketoglutaric acid (Lockwood and Stodola, 1946). The failure of all previous investigators except Alsberg to observe these phenomena may be ascribed to the use of weakly buffered and insufficiently aerated media (e.g., the customary tubes of carbohydrate broth), since acidity and poor oxygen supply are both limiting factors for the transformations in question.

The oxidation of monosaccharides to the corresponding -onic acids is a pattern of biochemical behavior that occurs elsewhere among bacteria, so far as is at present known, only in the *Acetobacter* group. Coupled with the frequently overlooked morphological similarities, it serves, as Vaughn (1942) has pointed out, to indicate a close relationship between the genera *Acetobacter* and *Pseudomonas*. Consequently it seemed of interest to find out whether the primary biochemical characteristic of the acetic acid bacteria, namely the oxidation of ethanol to acetic acid, might not also exist in the genus *Pseudomonas*.

## MATERIALS AND METHODS

Thirteen strains of fluorescent pseudomonads were studied, of which one was a strain of *Pseudomonas aeruginosa* and the remainder belonged to the *Pseudomonas fluorescens* species-group.<sup>1</sup> Three cultures (designated by the prefix NRRL) were received from Dr. Lockwood, by whom they had been used in studies on the metabolism of monosaccharides. The others (designated by the prefix A.3.) were isolated locally from soil, using the customary enrichment methods (den Dooren de Jong, 1926).

<sup>1</sup> The term "*P. fluorescens* species-group" is used to designate pseudomonads producing a fluorescent pigment but devoid of accessory phenazine pigments (pyocyanin, chlororaphin, etc.). The taxonomic criteria in current use with this group are inadequate, in my opinion, to justify any further specific subdivisions.

Ability to use ethanol as sole carbon source was tested by streaking on mineral agar plates (0.1 per cent  $\text{NH}_4\text{Cl}$ , 0.1 per cent  $\text{K}_2\text{HPO}_4$ , 0.05 per cent  $\text{MgSO}_4$ , and 1.5 per cent agar) containing 1.0 per cent ethanol, and comparing growth with that on a control plate devoid of carbon source. Preliminary observations on acid production from ethanol were made by streaking on mineral or peptone agar plates containing ethanol and  $\text{CaCO}_3$  and noting the formation of cleared zones in the carbonate around the bacterial growth. This method is also extremely useful for a rough screening of strains that produce acid from sugars.

For quantitative studies on ethanol oxidation, the organisms were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium. Incubation was at 30 C on a shaking machine. The medium consisted of 0.5 per cent Difco peptone with various concentrations of ethanol and, in some experiments, also 0.5 per cent  $\text{CaCO}_3$ .

TABLE 1  
*Acetic acid production from ethanol by strains of the*  
*P. fluorescens* *species-group after 5 days*

STRAIN	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED	YIELD OF ACETIC ACID*
	mg	mg	mg	
Uninoculated	536			
A.3.1	82	454	58	10
A.3.2	0	536	242	35
A.3.3	21	515	145	21
A.3.6	0	536	313	45
A.3.8	152	384	365	71
A.3.9	0	536	45	6
A.3.10	48	488	71	11
NRRL B-13	24	512	5	1

Medium: 0.5 per cent peptone, 0.5 per cent  $\text{CaCO}_3$ , and 1.0 per cent ethanol.

\* Expressed as percentages based on ethanol oxidized.

Ethanol was determined by dichromate oxidation of neutral distillates and estimation of residual dichromate; acetic acid, by titration of steam distillates. The acetic acid was identified by the iodine-lanthanum reaction and by formation of the characteristic copper salt (Meyer, 1933, p. 101).

#### RESULTS

Nine of the 13 strains were capable of developing abundantly on mineral, ethanol agar with ethanol as the sole carbon source. The remaining 4 (including two—NRRL B-14 and B-25—received from Dr. Lockwood) failed to develop on this medium. Of the 9 positive strains, 7 produced sufficient acid on mineral, ethanol,  $\text{CaCO}_3$  agar to cause a marked dissolution of the carbonate, and one more (NRRL B-13) produced a very slight amount of acid. The only ethanol-utilizing strain which failed to produce any acid whatsoever was the isolate of *P. aeruginosa*. Ethanol also gave rise to acid production when the mineral base

was replaced by 0.5 per cent peptone; indeed, under these conditions slightly more acid appeared to be formed.

Quantitative data on ethanol oxidation and acetic acid formation by the 8 acid-producing strains are shown in table 1. The medium contained 0.5 per cent  $\text{CaCO}_3$  and slightly over 1 per cent ethanol. It can be seen that the degree of acetification varies very greatly from strain to strain. Some carry out a virtually complete oxidation of the ethanol with negligible accumulation of

TABLE 2  
*Total titratable acidity and final pH produced by three strains of fluorescent pseudomonads when grown in 50 ml of peptone, 1.5 per cent ethanol broth*

STRAIN	TITRATABLE ACIDITY, ML OF 0.1 N			FINAL pH
	36 hr	60 hr	84 hr	84 hr
Uninoculated	2.0	2.0	2.0	7.35
A.3.1	1.5	8.5	8.5	4.55
A.3.2	1.3	10.1	10.1	4.50
A.3.8	7.0	7.0	7.0	4.95

Cultures grown at 30 C on a shaking machine.

TABLE 3  
*Acetic acid production from ethanol by strain A.3.8*

MEDIUM*	FINAL pH	TITRATABLE ACIDITY ML OF 0.1 N	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED
			mg	mg	mg
Peptone, control.....	7.15	1.6			
Peptone, inoculated.....	8.80	0.0			
Peptone, ethanol, control.....	7.15	1.8	535		
Peptone, ethanol, inoculated.....	4.55	10.0	276	259	50
Peptone, ethanol, $\text{CaCO}_3$ , control.....	7.75		555		
Peptone, ethanol, $\text{CaCO}_3$ , inoculated.....	5.00		57	498	231

Cultures were grown for 5 days at 30 C on a shaking machine.

\* Constituents of the medium were used in the following concentrations: peptone, 0.5 per cent; ethanol, 1.0 per cent; and  $\text{CaCO}_3$ , 0.5 per cent.

acetic acid, whereas others convert a substantial proportion of the ethanol oxidized into acetic acid.

In the absence of  $\text{CaCO}_3$ , acid production (as gauged by titratable acidity) is slight, even with the most actively acetifying strains. This is owing to the fact that the pH soon drops below 5.0 and the organisms die off. Typical figures for titratable acidities and final pH in peptone ethanol broth cultures are given in table 2. Streaked plates made from such cultures after 3 to 4 days reveal the presence of very few viable cells. A somewhat more detailed picture of the effect of  $\text{CaCO}_3$  addition is given for strain A.3.8. in table 3.

## DISCUSSION

The present demonstration that some fluorescent pseudomonads can produce substantial amounts of acetic acid from ethanol might have been predicted in the light of recent work on their metabolism. Although acetification is not a universal property of these organisms, some strains being unable to attack primary alcohols at all, its very existence in the *P. fluorescens* species-group raises a nice taxonomic problem, since the family *Acetobacteriaceae* and the genus *Acetobacter* are currently segregated from other pseudomonads primarily on the basis of their ability to produce acetic acid from ethanol. In view of the extensive morphological and biochemical parallelism between acetic acid bacteria and organisms of the *P. fluorescens* type, it seems indefensible any longer to maintain a family *Acetobacteriaceae*; its members should be incorporated in the family *Pseudomonadaceae*. The genus *Acetobacter*, if it is to be kept at all, must be redefined in a manner which no longer stresses so exclusively the fact of acetification. As an additional differential property, acid tolerance, which is so marked in these organisms as contrasted with other heterotrophic pseudomonads, should be considered.

## SUMMARY

Certain strains of the *Pseudomonas fluorescens* species-group can oxidize ethanol with the production and accumulation of acetic acid. The intensity of acetification varies greatly from strain to strain. Acetification proceeds best in a medium well buffered with calcium carbonate. In poorly buffered media, ethanol oxidation is soon checked by increasing acidity.

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# GROWTH RESPONSES OF A SULFONAMIDE-REQUIRING MUTANT STRAIN OF NEUROSPORA<sup>1</sup>

STERLING EMERSON<sup>2</sup>

*Wm. G. Kerckhoff Laboratories of the Biological Sciences, California Institute of  
Technology, Pasadena, California*

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A mutant strain of *Neurospora crassa* has appeared in which the antagonistic roles of *p*-aminobenzoic acid and the sulfonamides have been reversed to a considerable extent. Optimal growth of this strain occurs only in the presence of sulfonamides. Conversely, *p*-aminobenzoic acid is a potent fungistatic agent for this strain under certain conditions.

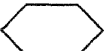
To say that sulfanilamide has become an essential metabolite and *p*-aminobenzoic acid an inhibiting analog would be to oversimplify the altered physiology of this mutant strain. It will be shown that, in this strain, both sulfonamides and *p*-aminobenzoic acid are essential for growth, and that each acts as an inhibiting analog of the other. These interrelations are further complicated by the effect of temperature on the need for sulfonamides, and on the inhibition by *p*-aminobenzoic acid.


The present report deals exclusively with the growth responses of this mutant strain to sulfonamides, to temperature, and to *p*-aminobenzoic acid. At the present time nothing definite is known of the physiological role of sulfonamides in this strain.

## MATERIALS AND METHODS

**Methods.** The procedures followed are essentially those described in a previous report (Emerson and Cushing, 1946). Growth responses are recorded as growth rates, which were determined by the tube method of Ryan, Beadle, and Tatum (1943).

**Symbols.** For the sake of clarity and brevity, the following symbols will be used:

PABA—*p*-aminobenzoic acid,  $\text{H}_2\text{N}$    $\text{COOH}$

SA —sulfanilamide,  $\text{H}_2\text{N}$    $\text{SOONH}_2$

pab —“*p*-aminobenzoicless,” a gene interrupting the synthesis of PABA, strain 1633 of Tatum and Beadle (1942).

+<sup>pab</sup> —the wild-type allele of pab.

sfo —“sulfonamide-requiring,” a gene carried by strain E-15172 described in this paper.

<sup>1</sup> Representing work supported in part by a grant from the Rockefeller Foundation, and in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>2</sup> With the technical assistance of Mary R. Emerson and Lydia Hawk.

- $+^{sfo}$  —the wild-type allele of *sfo*.  
 S-T —“sulfanilamide tolerant,” a gene for resistance to SAN, strain C-40 (Emerson and Cushing, 1946).  
 $+^{S-T}$  —the wild-type allele of S-T.

*Origin of sulfonamide-requiring strain.* In a previous communication (Emerson and Cushing, 1946) mention was made of a mutant strain (E-13190) which apparently required sulfonamides for growth. Mutant E-13190 appeared as a segregant in one ascus of a cross between the sulfanilamide-tolerant strain and a wild-type strain [C-40(E-8577)A  $\times$  E-5297a].

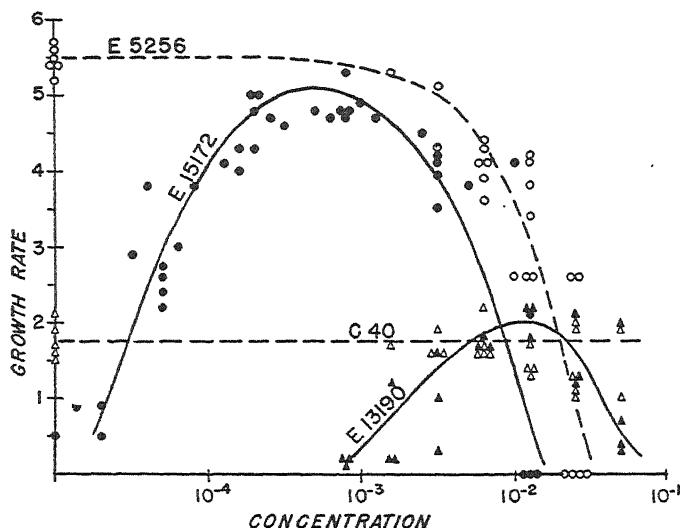


FIG. 1. GROWTH RATES (IN MILLIMETERS PER HOUR) OF FOUR GENETICALLY DIFFERENT STRAINS ON VARYING CONCENTRATIONS OF SULFANILAMIDE

AT 35 C

E-5256, wildtype ( $+^{sfo} +^{S-T}$ ); E-15172, sulfonamide-requiring strain (*sfo*  $+^{S-T}$ ); C-40, sulfanilamide-tolerant strain ( $+^{sfo}$  S-T); E-13190, double mutant, sulfanilamide-tolerant and sulfonamide-requiring (*sfo* S-T).

Mutant strain E-13190 proved to be a “double mutant” carrying the gene for sulfanilamide tolerance (S-T) characteristic of strain C-40 as well as the new mutant gene (*sfo*) for sulfonamide requirement. In an outcross of strain E-13190A to wild type (Abb-12a) these two genes segregated independently. The gene responsible for sulfonamide requirement was isolated from this cross as E-15172A. The four different genetic constitutions resulting from this cross are identified by their responses to varying concentrations of SA (figure 1). The double mutant (*sfo* S-T) has the maximal growth rate of about 2 mm per hour characteristic of the S-T strain and requires about 50th molar SA for optimal growth at 35 C. By itself *sfo* has a maximal growth rate of over 5 mm per hour, similar to that of wild-type, and grows optimally on a much higher dilution of SA.

Genetic tests show that *sfo* lies very close to the centromere of a different chromosome from that carrying *S-T*. Both these genes are independent of *pab*, which is located some distance from the centromere of an undetermined chromosome.

#### RESULTS

*Substances stimulating growth in strain E-15172.* The sulfonamide-requiring strain is able to utilize each of the sulfonamides that have been tested (figure 2) though the concentration necessary for optimal growth is different for different drugs. Growth was also supported by *p*-sulfamido-phenylalanine,<sup>3</sup> but never to maximal extent, perhaps because of inhibition resulting from competition between this analog and phenylalanine (cf. Mitchell and Niemann, 1947).

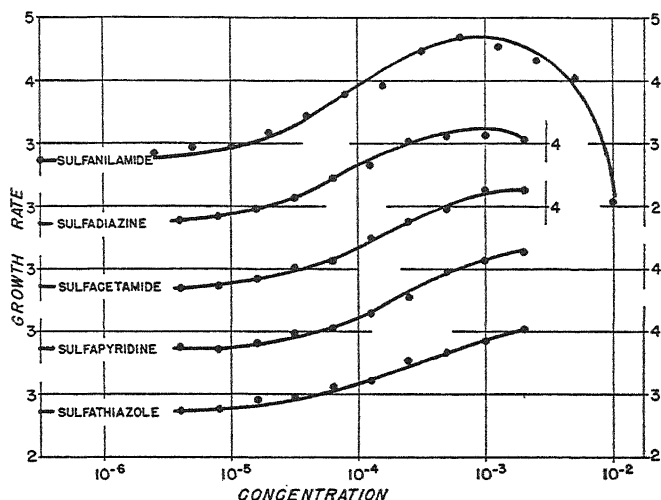


FIG. 2. GROWTH RATES (IN MILLIMETERS PER HOUR) OF SULFONAMIDE-REQUIRING STRAIN *SFO*, ON VARYING MOLAR CONCENTRATIONS OF DIFFERENT SULFONAMIDES AT 30 C. See discussion on temperature effect.

Methionine, the sulfone and sulfoxide of methionine, and taurine were unable to support growth of this strain, though methionine and its sulfoxide are utilized by certain other strains which require an organic source of sulfur (Horowitz, unpublished).

*Effect of temperature on sulfonamide requirement.* Although the sulfonamide-requiring strain will not grow at 35 C unless sulfonamides are present, considerable growth occurs at lower temperatures in the absence of sulfonamides. Data from experiments in which SA concentration and temperature were varied simultaneously are summarized in a contour graph in figure 3. In this diagram SA concentration increases from about millionth molar at the left to hundredth molar at the right. Temperatures increase from 25 C at the bottom of the diagram to over 36 C at the top. The contour lines pass through intersections of temperatures and concentrations at which equal growth rates occur.

<sup>3</sup> The *p*-sulfamido-phenylalanine was kindly supplied by Professor Carl G. Niemann.

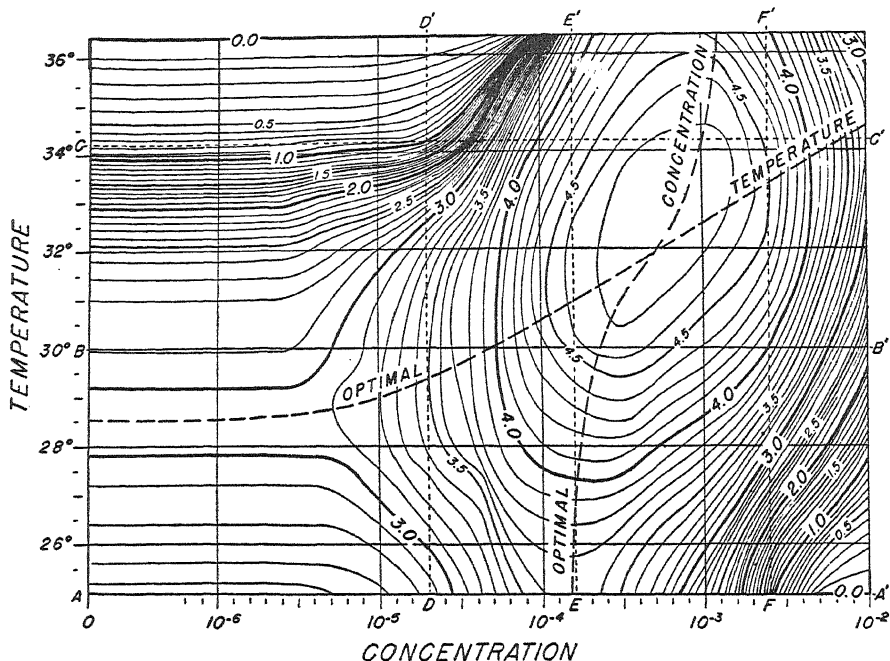


FIG. 3. GROWTH RATES OF SULFONAMIDE-REQUIRING STRAIN, SFO, WITH VARYING TEMPERATURE AND SA CONCENTRATION

Contour lines pass through points having equal growth rates (expressed as millimeters per hour). Concentrations are expressed as moles per liter. Rates were determined at 25, 27.8, 30, 32, 34.2, and 36.4 C, and at twofold dilutions from  $m/100$  to  $m/1,638,400$ , for a total of 96 different combinations of temperature and concentration, one quarter of which were run in duplicate.

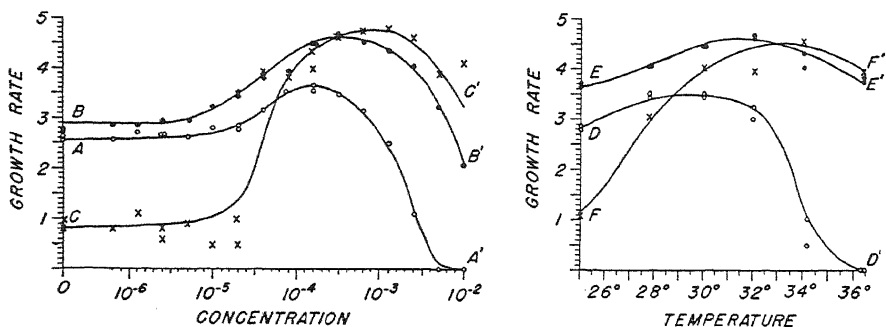


FIG. 4. GROWTH RATES OF THE SULFONAMIDE-REQUIRING MUTANT, SFO, ON VARYING CONCENTRATIONS OF SA (AT LEFT), AND AT VARYING TEMPERATURE (AT RIGHT)

Curve A-A', 25 C; B-B', 30 C; C-C', 34.2 C. Curve D-D',  $m/51,200$ ; E-E',  $m/6,400$ ; F-F',  $m/400$ . The curves represent sections through the graph in figure 3 along the lines A-A', B-B', E-E', etc. The points represent observed values.

Sections through this graph parallel to the base give curves showing the variations in growth rates with changing SA concentration at constant temperatures. Three such sections are reproduced in figure 4. Sections parallel to the sides of





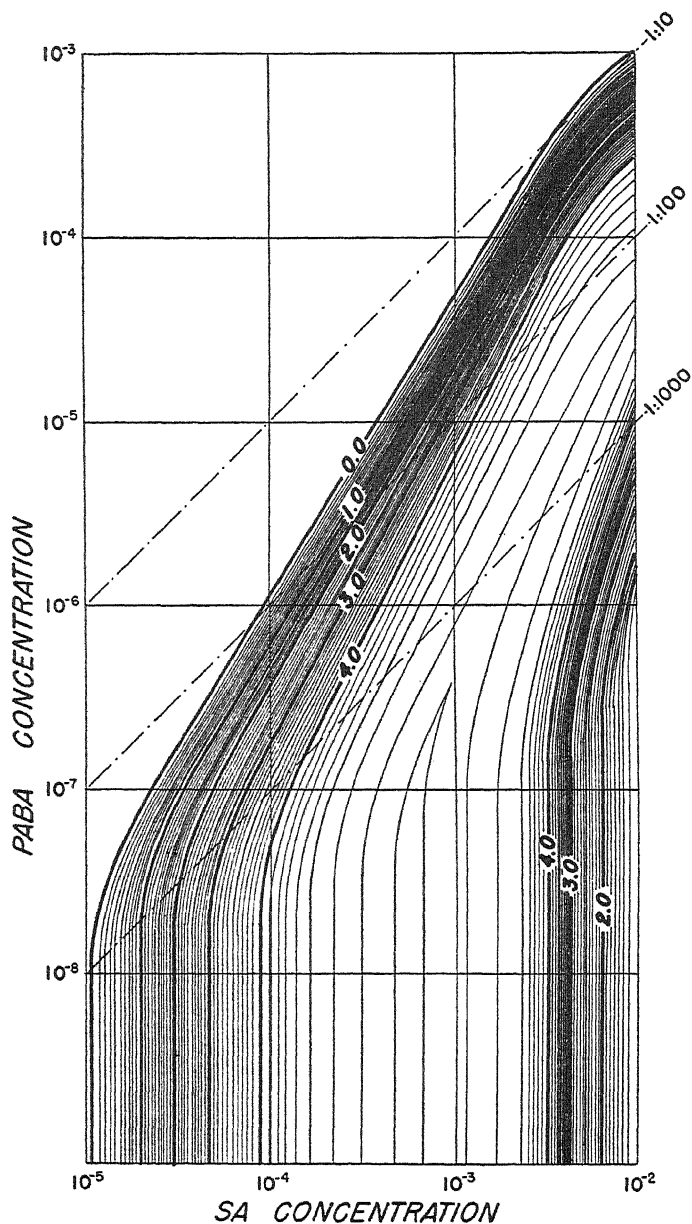


FIG. 6. CONTOUR GRAPH SHOWING GROWTH RATES OF THE SULFONAMIDE-REQUIRING STRAIN, SFO, AT 35 C ON VARYING MOLAR CONCENTRATIONS OF SA AND PABA

Growth rates are indicated by the contour lines. The ratios in the upper right margin are the molar ratios of PABA to SA. Based on determinations at 60 different combinations of SA and PABA concentrations.

luxuriant, and has a sharply defined frontier; such growth is represented by the

Conidial transfers from the ends of growth tubes showing reverted growth (designated by arrows in figure 5) indicated that the reversions were persistent for the most part (see discussion of persistent "adaptive" changes in Emerson

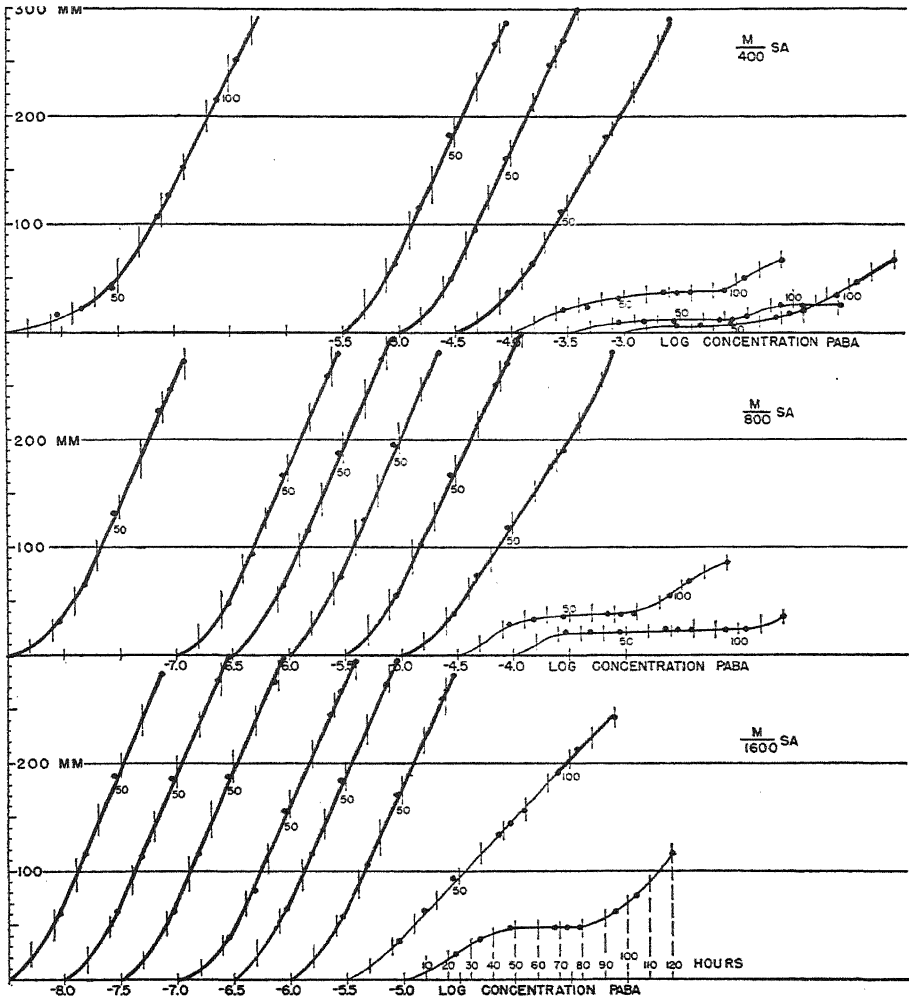


FIG. 7. FAMILIES OF GROWTH CURVES OF *sfo* STRAIN ON VARYING CONCENTRATIONS OF PABA.

Upper set of curves in the presence of  $M/400$  SA; middle set,  $M/800$ ; lower set,  $M/1,600$ . Positions of the origins of the curves along the base line indicate the concentrations of PABA for each. In each curve, horizontal distance represents time elapsed in hours, vertical distance represents total growth in millimeters. Heavy lines represent mycelial growth possessing definite frontiers, lighter lines represent "feathery" growth, with no well-defined frontier.

and Cushing, 1946). Transfers from such tubes to fresh tubes containing no SA generally resulted in growth resembling that of wild-type without any preliminary "feathery" stage such as is characteristic of *sfo*. Furthermore, crosses

from such reverted cultures generally showed that reversion had been accompanied by mutation at a locus distinct from that responsible for sulfonamide requirement. The fractions at the tops of growth curves in figure 5 show the num-

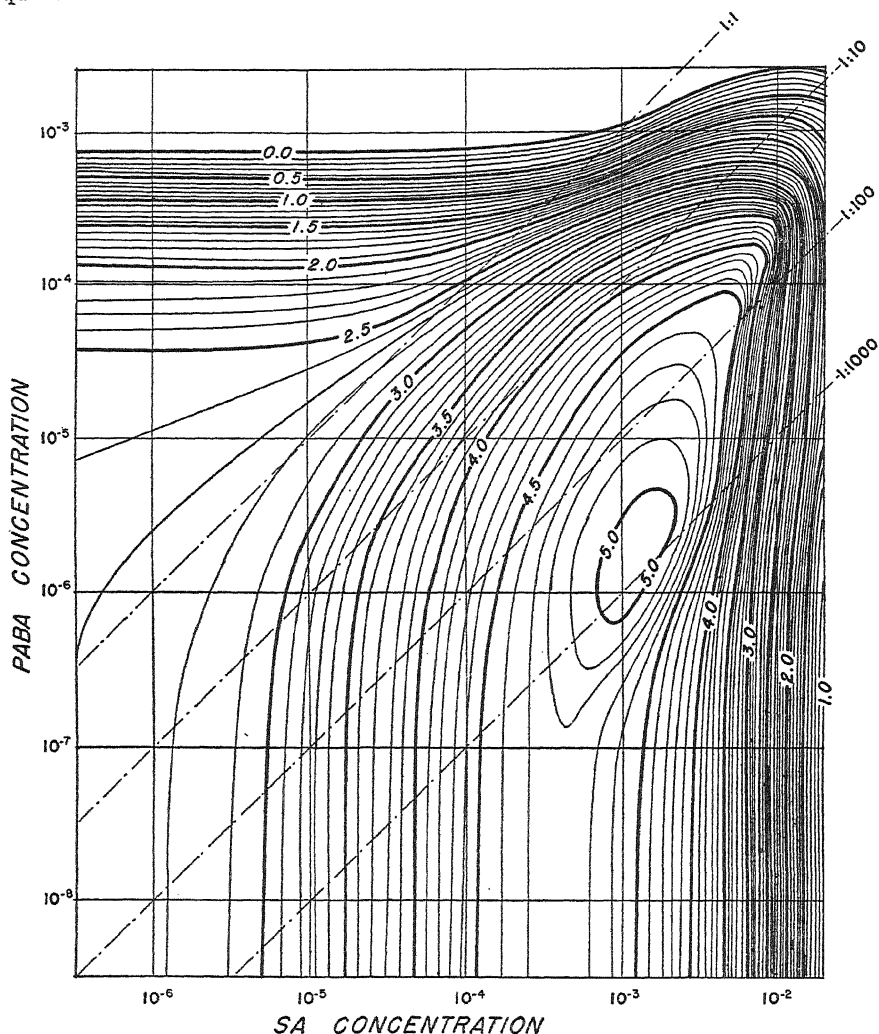


FIG. 8. CONTOUR GRAPH SHOWING GROWTH RATES OF SFO STRAIN AT 30°C IN THE PRESENCE OF VARYING AMOUNTS OF PABA AND SA

See legend to figure 6. Based on 144 determinations at 96 different combinations of SA and PABA concentrations.

ber of nuclei carrying such mutations in the total nuclei tested from each culture. It follows that these mutations are not strictly reversions, but rather suppressions of the effects of sfo by another gene.

Since the more rapid "reverted" growth is presumably always the result of an

altered genetic constitution, the rates obtaining before reversions occur are taken as characteristic of sfo.

*Competitive inhibition of growth by p-aminobenzoic acid.* Under conditions which make sulfonamides essential for growth of the sulfonamide-requiring strain, PABA inhibits growth in very low concentrations. When grown at 35 C in the presence of optimal or suboptimal concentrations of SA, PABA will inhibit growth at concentrations as low as millionth molar (figure 6). In the presence of an excess of SA, however, relatively small amounts of PABA are beneficial, though inhibition still occurs at higher concentrations. The stimulating effect of PABA at high SA concentration is principally in shortening the lag phase resulting from the toxicity of SA, as illustrated in figure 7, though the final growth rate may also be increased.

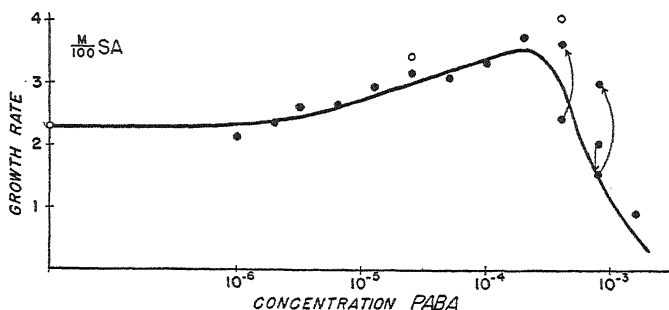


FIG. 9. GROWTH RATES OF SFO STRAIN AT 30 C IN THE PRESENCE OF M/100 SA AND VARYING MOLAR CONCENTRATIONS OF PABA

Growth rates expressed as millimeters per hour. Open circles represent data from one experiment, solid circles from another. Dots connected by arrows represent growth rates which changed during the course of growth down the tube (cf. figures 5 and 7).

At lower temperatures, at which sulfonamides stimulate growth but are not essential for growth, the inhibitory effect of PABA is very much less (figure 8). Relatively high concentrations of PABA are necessary for growth inhibition, and the inhibiting concentration is relatively independent of the amount of SA present. On the other hand, PABA does interfere with growth stimulation by SA. Maximum response to SA occurs only when the molar ratio of PABA to SA is less than 1 to 100. Here again in the presence of excessive amounts of SA, PABA partially overcomes the inhibition caused by the SA (figure 9).

*The simultaneous requirement of sulfanilamide and p-aminobenzoic acid by a double mutant.* The sulfonamide-requiring strain was crossed to the pab strain of Tatum and Beadle (1942), which requires PABA for growth, and the double mutant (sfo pab) isolated.<sup>4</sup> At 35 C this double mutant requires both PABA and SA (figure 10). Over most of the range of concentrations supporting growth

<sup>4</sup> Tatum and Beadle's pab strain 1633A was first crossed to wild-type strain E-5297a and the pab gene isolated free from an undesirable gene (temperature sensitive on lactose, etc., see Emerson and Cushing, 1946) as strain E-15835a, which was then crossed to sfo E-15172A. The double mutant, sfo pab, was isolated from this cross in strains E-16608A and E-16613a.

of the double mutant, a molar ratio of about 1 PABA to 1000 SA is most favorable. An excess of either analog is inhibitory in a competitive manner.

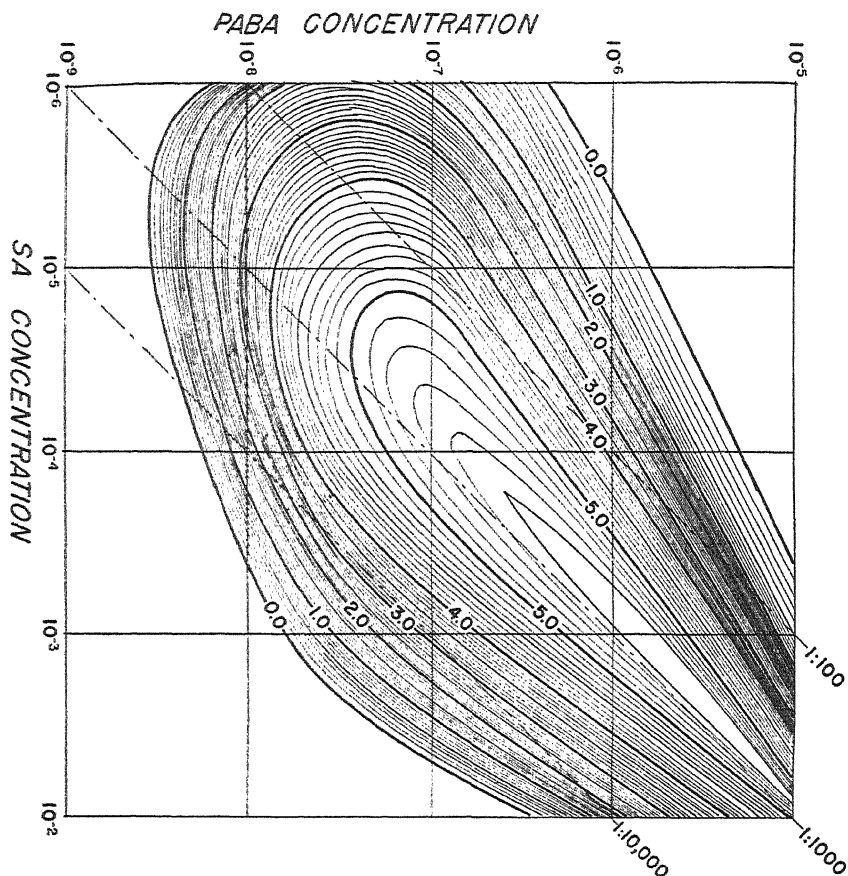


FIG. 10. CONTOUR GRAPH SHOWING VARIATION IN GROWTH RATE WITH CHANGING CONCENTRATIONS OF PABA AND SA OF THE DOUBLE MUTANT (sfo PAB) AT 35 C

See legend to figure 6. Based on 84 determinations at 52 combinations of SA and PABA concentrations.

#### DISCUSSION

*Sulfanilamide as a metabolite.* Although growth responses by themselves do not prove that a substance found to be necessary for growth is actually used as a metabolite, the data just reported make it seem highly probable that sulfanilamide is so utilized by strain E-15172 (sfo). In the first place, of the substances tested only sulfonamides were capable of supporting growth of the sfo strain at 35 C. Secondly, this strain does not produce excessive amounts of PABA and thus require sulfonamides as antagonists because: (1) Inhibition of wild-type *Neurospora* by PABA is not antagonized by SA (Emerson and Cushing, 1946).

(2) The double mutant *sfo pab* cannot synthesize PABA and needs both SA and PABA for growth at 35 C. In the double mutant there can be no question of an overproduction of PABA, yet sulfonamides are still required. Thirdly, the competitive inhibition of growth of the *sfo* strain by PABA suggests that the structurally similar SA is actually used as a metabolite.

*Sulfanilamide-p-aminobenzoic acid ratios.* In most instances of competitive growth inhibition the molar ratio of inhibiting analog to metabolite is rather large. The competitive inhibition of wild-type *Neurospora* by SA is of this sort (Tatum and Beadle, 1942; Emerson and Cushing, 1946). It is all the more striking, therefore, that in the PABA inhibition of the sulfonamide-requiring strain the ratio of inhibiting analog to "metabolite" is just reversed, being about 1 PABA to 100 SA.

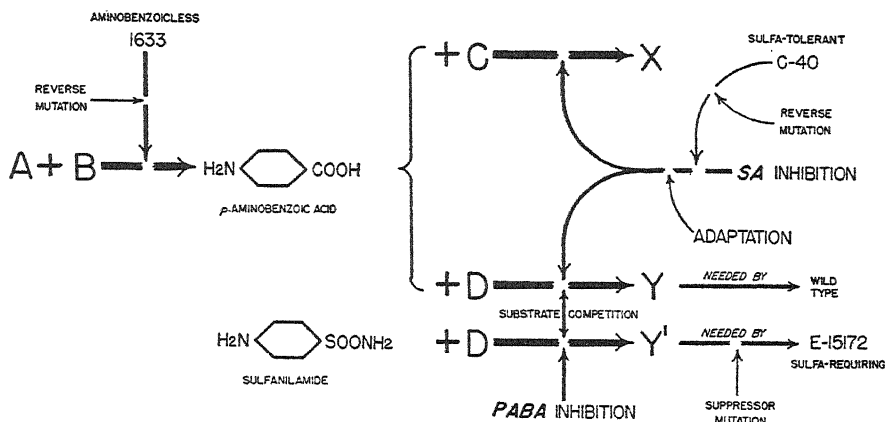


FIG. 11. FORMAL SCHEME TO SUMMARIZE THE INTERPLAY OF MUTATIONS EFFECTING SA-PABA RELATIONSHIPS

Heavy arrows represent enzymatic reactions; breaks in arrows represent interruptions due to genetic blocks or to substrate inhibitions.

Since the molar ratio of PABA to SA is the same regardless of which is the inhibiting analog, one is tempted to suggest that the same enzymatic reaction is involved in both cases. If two substrates (PABA and SA) compete for the same enzyme, and if both are transformed by that enzyme, the relative amounts of the two products resulting will depend upon the relative amounts of the two substrates. Then if wild-type requires one of these end products (Y in figure 11), and the sulfonamide-requiring mutant the other (Y'), the ratios of inhibiting analog to metabolite should be reversed as one metabolite is replaced by the other, just as reported above.<sup>5</sup>

A formal scheme giving a pictorial summary of the interplay of the various

<sup>5</sup> Nearly everyone with whom I have discussed this case has suggested that the sulfonamide requirement of strain E-15172 might be accounted for by some such scheme. I believe that the particulars just outlined were first suggested by my collaborator Dr. Marko Zalokar.

mutations studied is given in figure 11. The aminobenzoicless mutant (pab) is known to interrupt the synthesis of PABA (Tatum and Beadle, 1942). In the absence of PABA the gene pab is sometimes changed to  $+^{pab}$  by reverse mutation, restoring the wild-type condition in which the synthesis of PABA continues normally.

It is supposed that PABA takes part in more than one essential reaction (e.g., with substances C and D in the diagram). This would be in agreement with the observations of Lampen *et al.* (1946), which suggest that PABA is concerned with three different sorts of syntheses. The inhibition of growth by SA is supposed to be due to substrate competition with PABA in one or more of these reactions. Such SA inhibition can be lessened by nongenetic adaptation (Emerson and Cushing, 1946), or largely overcome by mutation to sulfanilamide tolerance (S-T). Especially in the presence of PABA or sulfathiazole, reverse mutation changes S-T back to wild-type ( $+^{S-T}$ ).

The sulfonamide-requiring mutant, sfo, is shown as differing from wild-type by needing the end product  $Y'$  in place of  $Y$ . As illustrated, the double mutant pab sfo, requiring both PABA and SA for growth, needs  $X$  as well as  $Y'$ . On this supposition, SA would interfere with the production of  $X$ , PABA with the production of  $Y'$ . It is also possible that in place of  $X$  and  $Y'$  the double mutant needs  $Y$  and  $Y'$ , say in approximately equal amounts. Again a balance between SA and PABA would be essential as the production of  $Y$  is inhibited by excess SA, of  $Y'$  by excess PABA.

"Reversions" of the sfo mutant to growth resembling wild-type are due to "suppressor" mutations. These are mutations of a gene distinct from sfo which suppress the sulfonamide requirement characteristic of sfo.

The scheme illustrated is meant simply as a convenient summary. Direct evidence of the role of SA in the metabolism of the sulfonamide-requiring mutant must await the chemical determination of the fate of SA in the organism.

#### SUMMARY

Mutant strain E-15172 requires sulfonamides for growth at 35 C. At 30 C or lower sulfonamides are not strictly essential, but growth rates are depressed without them.

At high temperatures (34 C or over) *p*-aminobenzoic acid inhibits growth of this strain at high dilutions ( $10^{-6}$  molar). Growth inhibition by *p*-aminobenzoic acid is competitively antagonized by sulfanilamide. The ratio of *p*-aminobenzoic acid to sulfanilamide giving 50 per cent growth inhibition is about 1:100.

A double mutant, carrying the gene for sulfonamide requirement and a gene for the failure of synthesis of *p*-aminobenzoic acid, requires both sulfonamides and *p*-aminobenzoic acid for growth. The molar ratio giving maximum growth at 35 C is about 1,000 sulfanilamide to 1 *p*-aminobenzoic acid.

The possibility that sulfanilamide is utilized by strain E-15172 as a metabolite is discussed.



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# THE RELATION OF THE BACTERIAL PRODUCTION OF AMMONIA GAS TO THE GROWTH OF OTHER MICROORGANISMS

FRANCIS J. RYAN AND LILLIAN K. SCHNEIDER

*Department of Zoology, Columbia University, New York 27, New York*

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In an experiment on the growth of *Neurospora crassa* a wide zone of inhibition was observed on the surface of agar surrounding a contaminating bacterial colony. The heat-resistant contaminant was isolated and labeled UB1. A few preliminary experiments indicated that the inhibition was of such a drastic nature as to warrant further investigation.

Because the 24-hour growth of UB1, after being streaked across an agar plate, completely inhibited the growth of several strains of *Neurospora* which were inoculated about 3 or 4 cm away, it was suspected that the inhibiting factor may be a gas. In order to test this point the following experiment was performed: The base of a petri dish, 7.5 cm in diameter, was sterilized inside a 10-cm petri dish so that two surfaces of agar completely separated by glass could be prepared as shown in figure 1. Such surfaces were prepared with Fries medium (Ryan, Beadle, and Tatum, 1943) containing 0.5 per cent "casamino" acids and 2 per cent agar. A culture of UB1 was streaked on surface A and allowed to grow at 25 C for 40 hours. A wild-type strain of *Neurospora crassa*, 1A, was then inoculated onto the agar contained in the central petri dish (B). Inhibition of mold growth was again observed, whereas control plates which had not been streaked with UB1 permitted luxuriant growth of *Neurospora*. Consequently, it was necessary to conclude that UB1 produced some substance which passed through the air over the edge of the inner petri plate.

In a similar fashion it was shown that 8 other strains of *Neurospora*, including biochemical mutants, could be inhibited by UB1. In one experiment, although *Neurospora* was inhibited, the agar in the central plate (B) possessed deep agar colonies of a new contaminating bacterium. In a control plate, which had not been inoculated with either UB1 or *Neurospora*, no such colonies appeared. One of these new contaminating colonies was isolated (called UB2), and cultures of it were introduced into the agar in region (B) of the double petri plate. Here it grew only when strain UB1 was streaked around it in region A. Thus strain UB1, in addition to producing a gaseous inhibitor of *Neurospora*, produces a gaseous substance which enables the growth of UB2. These gases may, of course, be the same.

The pH of the agar medium we had been using was 5.6 and optimum for the growth of *Neurospora*. It was observed in some control plates, which contained UB1 in region A but no other organism, that the pH of the central agar rose to between 7 and 8. Consequently, UB1 produces an alkaline gas which can raise the pH of the agar. This accounts for the inhibition of *Neurospora*, whose rate

of growth decreases rapidly with a change in pH from 6.5 to 8.0 (Ryan, Beadle, and Tatum, 1943). In order to determine whether the change in pH of the agar was also responsible for the growth of UB2, this strain was grown in liquid 0.5 per cent casamino acids (Fries) at a series of different hydrogen ion concentrations. At pH's of 5, 6, and 9 it failed to grow; only pH's of 7 and 8 supported growth. This property accounts for the stimulation of the growth of UB2 by UB1, but the nature of the alkaline gas produced by the latter organisms remained to be determined.

Since UB1 also changes the pH of the medium on which it grows to about 8, ammonia gas can be suspected as the agent. In the following experiments, the agar in region A of the double plates was brought to pH 7 because better growth of UB1 occurred there than at pH 5.6. Also for better growth a temperature of 37 C was used. All these experiments were controlled by double plates containing UB1 in region A alone, UB2 in region B alone, UB1 in region A and UB2 in region B, and, finally, no organisms in either region. It was found that raising the pH of the central agar in B to 7 by either sodium hydroxide or ammonium hydroxide resulted in the growth of UB2 in the absence of UB1. Moreover, when the central agar containing UB2 was left at pH 5.6 and ammonium

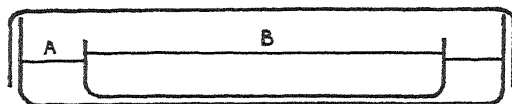


FIG. 1. OPTICAL SECTION OF THE ARRANGEMENT OF ONE PETRI DISH INSIDE ANOTHER, ENABLING THE PREPARATION OF AGAR SURFACES SEPARATED BY GLASS

hydroxide, instead of agar and UB1, was placed in region A, abundant growth ensued. Ammonia gas is therefore able to account for the experimental results.

In order to demonstrate that UB1 produces gaseous ammonia, a culture was streaked on the agar in region A, while region B, instead of agar and UB2, contained 10 ml of 0.05 N sulfuric acid. After UB1 had grown for 24 hours, the sulfuric acid solution was examined for ammonia with Nessler's reagent. About 1.22 mg of ammonia was found, whereas about 1.3 mg are required to raise the pH of the buffered Fries medium from 5.6 to 7.5. It therefore appears that the gaseous ammonia produced by UB1 is sufficient to account both for the stimulation of UB2 and the inhibition of *Neurospora*.

The ammonia produced by UB1 is undoubtedly derived from the amino acids in the casamino medium. UB1 will not use nitrate or ammonium ions as a nitrogen source, nor will UB2. The latter strain, at a pH of 5.6, will not use amino acids either, but will grow at that pH if tryptose is added. These two strains of bacteria differ in other respects; UB1 grows much more vigorously on all the media we have tried, it forms a pellicle on liquid, and its cells are shorter and thinner than those of UB2 (length of UB1, 2 to 2.5  $\mu$ ; of UB2, 3 to 4  $\mu$ ). Both strains, however, consist of motile, aerobic, gram-positive rods which form central spores. These spores are, in both cases, very resistant to heat and will withstand boiling for 10 minutes. In addition, UB1 will grow on tryptose at

55 C, although apparently no better than at 37 C. The two strains have not been characterized further but appear to belong to the *Bacillus subtilis* group. The production of ammonia by members of this group has been reported by Cook and Woolf (1928).

#### SUMMARY

A bacterial strain, secured from a plate contaminant, is able to produce ammonia gas in such amounts as to change the pH of buffered agar some distance away. This behavior can result in the complete inhibition of the growth of the mold, *Neurospora crassa*, and, in addition, can enable the growth of a second strain of bacteria with a demanding pH requirement.

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# THE GROWTH AND PIGMENTATION OF ACTINOMYCES COELI-COLOR AS AFFECTED BY CULTURAL CONDITIONS

VINCENT W. COCHRANE AND JEAN E. CONN

Connecticut Agricultural Experiment Station, New Haven, Connecticut, and Biological Stain Commission, Geneva, New York

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The use of characteristic pigments as criteria in species differentiation among the actinomycetes has been hampered by the comparative lack of knowledge of the conditions under which these pigments are produced. The striking red-blue indicator pigment of *Actinomyces coelicolor* (Müller) Lieske has been described (Conn, 1943) as a possible taxonomic character. The present investigation was designed to determine the cultural conditions which affect the formation of this pigment, with particular regard to carbon nutrition, nitrogen nutrition, and pH relations.

## MATERIALS AND METHODS

The culture of *A. coelicolor* used has been described by one of us (Conn, 1943) as isolate B-3. The basal medium used throughout contained cp glucose, 10 g per L; Difco asparagine, 0.5 g per L; cp  $K_2HPO_4$ , 0.5 g per L; cp  $MgSO_4 \cdot 7H_2O$ , 0.25 g per L; Difco yeast extract, 0.5 g per L; and a minor element mixture composed of salts of Zn, Fe, Cu, B, Mn, and Mo in the amounts recommended by Robbins and Ma (1942). In this medium the sugar was found to be 85 to 90 per cent utilized in 18 days at 25 C; amounts of asparagine in excess of 0.5 g per L did not increase dry weight with glucose at 10 g per L. The organism was grown on the surface of 50 ml of liquid medium in 125-ml Erlenmeyer flasks. In still culture this and other actinomycetes make normal growth only on the surface; the flocculent subsurface mycelium so often noted in liquid cultures is characterized by a very slow and irregular growth rate and by poor sugar utilization.

In experiments on carbon and nitrogen nutrition, replicate cultures were harvested at 6, 12, and 18 days after seeding; data from the last period only are reported. The dry weight was determined by drying the mycelium overnight at 70 C on tared filter paper. All pH measurements were made with the glass electrode.

To compare pigment production under different conditions, the filtered culture fluid was adjusted to pH 7.0 and diluted with 4 volumes of phosphate buffer (pH 6.98). The intensity of pigmentation was then read in a Fisher electrophotometer against a similarly adjusted and diluted sterile medium, using a filter with peak transmittance at 650m $\mu$ .

All cultures were run in triplicate. Dry weight determinations were made for each flask; other measurements were made on a pooled sample from the three replicates.

## CARBON AND NITROGEN NUTRITION

A condensed summary of data on the utilization of carbon and nitrogen sources is presented in table 1. Dry weight and pigment intensity were recalculated on the basis of glucose controls in the carbon source experiment, of asparagine controls in the nitrogen series.

Except with organic acids, unavailability of a particular carbon source was evidenced not only by low relative growth but by a high final pH, resulting from the utilization of asparagine as a source of energy. Examples of this were the media having as carbon source sorbose, inulin, dulcitol, and sorbitol. With neutralized organic acids as energy sources, a rise in pH was in many cases evidence of utilization.

Study of the growth data indicates that xylose, mannose, and glycerol were better carbon sources than glucose; arabinose proved to be much poorer, probably because of the production of acid. Sucrose was only slightly utilized; trehalose and lactose were slowly available. Among the organic acids, acetic, lactic, fumaric, succinic, malic, and gluconic acids were able to support growth in excess of that in the no-carbon control. Tartaric and citric acids did not support measurable mycelial growth, although the former was evidently attacked sufficiently to cause an increase in alkalinity.

Pigment formation was proportional to mycelial growth with one general exception: when the final pH was either high or low, pigment intensity was not so great as would be expected from the dry weight. This is particularly notable among the organic acids; in the poorly buffered basal medium the utilization of neutralized lactic, fumaric, succinic, and malic acids resulted in final pH values above 8.7, and no pigment was formed. Utilization of acetate and gluconate was not accompanied by such extreme alkalinity, and some pigment developed. A further exception to the general proportionality of growth and pigmentation was noted in glycerol media. The greater relative pigment intensity was not the result of reaction changes; it appears that glycerol specifically favors the production of pigment.

Turning to the nitrogen nutrition data of table 1, it is evident that all of the four amino acids tested were utilizable; urea also proved an adequate source of nitrogen. On the other hand, neither nitrate nor ammonium salts supported growth comparable to that with asparagine; in the case of nitrate, the dry weight was no higher than in the glucose yeast extract control. With ammonium salts, the low final pH is the cause of poor growth; this acidity results undoubtedly from preferential absorption of the ammonium ion. Supplementary experiments with ammonium phosphate at several concentrations showed that satisfactory growth occurs if the pH can be held above 6.0. In a poorly buffered medium it is impossible to supply enough ammonium nitrogen for utilization of 1 per cent glucose without the development of too acid a reaction. Failure of growth in the nitrate medium may be associated with the observed heavy accumulation of nitrite.

The several peptones tested supported excellent growth and pigmentation. The latter fact shows that so-called "synthetic" media are not necessary for the



formation of the red-blue indicator pigment. As in the case of carbon sources, the intensity of pigmentation was roughly proportional to growth except in media with an unfavorable reaction. This is not always clear from the 18-day

TABLE 1  
*The utilization of carbon and nitrogen sources by A. coelicolor*

CARBON SOURCE*	CON- CENTRA- TION	RELA- TIVE GROWTH†	RELA- TIVE PIGMENT INTEN- SITY†	FINAL pH	NITROGEN SOURCE‡	CON- CENTRA- TION	RELA- TIVE GROWTH§	RELA- TIVE PIGMENT INTEN- SITY§	FINAL pH
	g/L					g/L			
None.....		17	0	8.4	None.....		30	32	6.7
d-Glucose.....	10.0	100	100	7.2	l-Asparagine.....	0.50	100	100	6.7
d-Mannose.....	10.0	202	200	7.0	Glycine.....	0.29	83	36	7.0
d-Galactose.....	10.0	84	97	7.1	l-Leucine.....	1.00	76	36	7.0
d-Fructose.....	10.0	79	96	7.0	l-Tryptophane...	0.78	98	82	6.9
d-Xylose.....	10.0	143	121	7.2	Urea.....	0.24	86	51	7.0
l-Sorbose.....	10.0	26	0	8.5	NaNO <sub>3</sub> .....	0.64	18	0	6.0
l-Arabinose.....	10.0	65	46	5.2	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> ....	0.50	57	0	5.7
Starch.....	10.0	107	87	7.0	Ammonium				
Inulin.....	10.0	32	0	8.5	acetate.....	0.58	18	0	5.6
Trehalose.....	10.0	90	38	8.1	Peptone.....	1.00	146	118	6.8
Cellobiose.....	10.0	81	95	6.7	Tryptone.....	1.00	91	106	7.0
Maltose.....	10.0	62	43	7.3	Casitone.....	1.00	116	100	7.0
Lactose.....	10.0	105	64	6.8	Peptidase.....	1.00	175	118	6.4
Sucrose.....	10.0	34	0	8.4	Casamino acids..	1.00	116	129	6.6
Glycerol.....	10.0	135	170	6.6	Sodium				
Mannitol.....	10.0	82	88	7.1	caseinate.....	1.00	72	53	6.9
Dulcitol.....	10.0	20	0	8.6	Gelatin.....	1.00	106	29	6.4
Sorbitol.....	10.0	27	0	8.2	Egg albumin.....	1.00	44	35	6.0
Acetic acid.....	5.0	33	33	8.4					
Lactic acid.....	5.0	60	0	8.8					
Fumaric acid.....	5.0	69	0	9.1					
Succinic acid.....	5.0	47	0	8.9					
d-Malic acid.....	10.0	60	0	8.9					
Tartaric acid.....	5.0	20	0	8.7					
Citric acid.....	5.0	24	0	8.2					
Gluconic acid.....	5.0	82	25	8.2					

\* Basal medium (g/L): asparagine—0.5, yeast extract—0.5, K<sub>2</sub>HPO<sub>4</sub>—0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O—0.25, and minor elements.

† Dry weight and pigment intensity of glucose control taken as 100.

‡ Basal medium (g/L): glucose—10.0, yeast extract—0.5, K<sub>2</sub>HPO<sub>4</sub>—0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O—0.25, and minor elements.

§ Dry weight and pigment intensity of asparagine control taken as 100.

|| Total nitrogen, 0.106 g/L.

data alone; thus, the relatively low pigment intensity of glycine and leucine media was associated with an acid reaction (about pH 6.0) earlier in the growth cycle. The same was true of the gelatin medium.

The data suggest that the nitrogen sources most favorable for growth and pigmentation—asparagine, tryptophane, and the peptones—are so not because

of the greater availability of nitrogen per se, but because the utilization of these materials is not accompanied by drastic pH changes. This, rather than specific nitrilite effects, may explain also the growth-promoting properties of yeast extract.

If this is indeed the case, an excess of any nitrogen source, if unbalanced by an increase in the glucose level, should cause a reaction unfavorable for pigment formation. The experiment summarized in table 2 affords confirmation of this hypothesis. Failure of pigment to develop in high peptone media was associated with a high pH, but there was no measurable reduction in growth. Experiments not reported in detail showed that the deleterious effect of high peptone levels on chromogenesis can be eliminated by increasing the concentration of glucose.

TABLE 2

*The effect of peptone concentration on growth and pigmentation of A. coelicolor\**

NITROGEN SOURCE	CONCENTRATION	pH		DRY WEIGHT	RESIDUAL SUGAR	COLOR†	PIGMENT INTENSITY
		Initial	Final				
	g/L			mg	mg/100 ml		
None.....		6.62	7.01	36.2	306.4	P	12.5
Asparagine.....	0.5	6.94	6.90	68.3	138.1	P	26.0
Peptone.....	0.5	6.86	6.99	54.0	303.2	P	23.0
Peptone.....	1.0	6.85	6.98	87.2	33.4	P	26.8
Peptone.....	2.0	6.88	7.69	83.1	8.4	B	39.3
Peptone.....	5.0	6.84	8.46	81.7	13.9	0	

\* Basal medium (g per L): glucose—10.0, yeast extract—0.5,  $K_2HPO_4$ —0.5,  $MgSO_4 \cdot 7H_2O$ —0.25, and minor elements. Duration of experiment 18 days.

† 0—none, P—purple, B—blue.

From these considerations it follows that the apparent utilization of a given source of nitrogen in part depends on the nature of the carbon source used and its concentration, since these factors affect the pH of the medium.

#### THE INFLUENCE OF pH ON GROWTH AND PIGMENTATION

Changes in reaction have been postulated as the explanation of several phenomena of nutrition, especially with regard to the utilization of organic acids, peptone, and ammonium salts. An experiment, summarized in table 3, tested the effect of variations in the initial pH of the culture medium on growth and pigment formation.

Growth occurred in media adjusted initially to pH 5.0 to 10.9, with the maximum dry weights at pH 6.9 to 7.7. The range in which pigment formed was somewhat narrower, pH 6.0 to 9.9, the optimum for pigment intensity being pH 7.3 to 7.7. It is evident that the organism is able substantially to lower the pH of alkaline media when glucose is the source of carbon; in order to obtain a final pH comparable to that attained in high peptone media, the medium had to be initially at pH 11.0. The difference between the pH limits of growth and

those of pigment formation explains the failure of pigmentation to accompany growth in media which become acid or alkaline during metabolism.

TABLE 3

*The influence of the pH of the medium on growth and pigment production of A. coelicolor\**

MEDIUM	pH		DRY WEIGHT	COLOR†	PIGMENT INTENSITY
	Initial†	Final			
			mg		
A	3.70	3.63	-0.2	0	
B	4.21	4.24	0.3	0	
C	4.98	4.92	27.5	0	
D	6.00	6.30	65.0	R	12.5
E	6.90	6.59	89.7	P	23.6
F	7.31	6.97	87.5	P	32.9
G	7.70	6.97	82.0	P	34.9
H	8.08	7.40	67.0	B	19.7
I	8.30	7.46	61.5	B	22.9
J	9.00	7.50	52.0	B	21.2
K	9.42	7.81	62.5	B	18.8
L	9.92	7.94	54.7	B	8.9
M	10.92	8.61	30.0	0	
N	11.20	11.06	0.3	0	

\* Basal medium (g/L): glucose—10.0, asparagine—0.5, yeast extract—0.5,  $K_2HPO_4$ —0.5,  $MgSO_4 \cdot 7H_2O$ —0.25, and minor elements. Glucose added aseptically after sterilization. Duration of experiment 18 days.

† Adjusted with HCl or NaOH, pH measured after sterilization.

‡ 0—none, R—red, P—purple, B—blue.

#### DISCUSSION

The controlling factor in the production of pigment by a vigorously growing culture of *A. coelicolor* is the reaction of the medium. Within the range which permits chromogenesis, the actual color of the pigment is again determined by pH. Of the variety of carbon and nitrogen sources tested, there was none which supported growth but not chromogenesis except those compounds the presence of which or the utilization of which caused the pH to drop below about 6.0 or to rise above about 8.5.

Within the pH range favorable for chromogenesis there is a discernible effect of reaction on the amount of pigment formed. The pH range for optimum production of pigment is somewhat narrower than the range of maximum mycelial growth. For this reason it is possible to effect changes in the amount of color without changing the final dry weight of the culture.

The possible taxonomic value of the pigments of actinomycetes has been discussed elsewhere (Conn and Conn, 1941; Conn, 1943). The present work emphasizes the need for careful control of certain environmental factors, and demonstrates that such control makes it possible to obtain reproducible results. Parenthetically, it may be mentioned that the strain of *A. coelicolor* used has been

carried in culture for 8 years with no detectable change in the pigment or in other characters.

#### SUMMARY

The growth of *Actinomyces (Streptomyces) coelicolor* Müller in surface culture has been studied with particular reference to the formation of the pigment characteristic of this species.

In a survey of carbon sources, mannose, xylose, and glycerol were found to support the heaviest mycelial growth. The organism is able to utilize a wide range of sugars, polyatomic alcohols, and organic acids. Compounds not utilized included sorbose, inulin, sorbitol, dulcitol, tartaric acid, and citric acid; sucrose is only slightly utilized.

Satisfactory nitrogen sources for growth include several amino acids and peptones, urea, casein, and gelatin. Ammonium salts of weak acids support normal growth only in a buffered medium; in a poorly buffered medium the acidity arising from preferential absorption of the ammonium ion interferes with growth. Nitrate is absorbed but undetermined secondary effects make it unsuitable under the conditions tested.

The optimum pH for growth is pH 6.9 to 7.7; the lower limit of growth is pH 4.2 to 5.0, the upper limit pH 11. Pigment is formed in media having an initial pH of 6.0 to 9.9, the optimum being pH 7.3 to 7.7.

Regardless of the specific compounds used to supply carbon and nitrogen, pigment develops in any medium able to support mycelial growth, provided that the course of metabolism does not shift the final reaction to either side of the range pH 6.0 to 8.5. Any medium, "synthetic" or not, which supports growth without drastic pH changes also supports chromogenesis.

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# BIOTIN AND THE SYNTHESIS OF ASPARTIC ACID BY MICROORGANISMS

J. L. STOKES, ALMA LARSEN, AND MARION GUNNESS

*Research Laboratories, Merck and Company, Inc., Rahway, New Jersey*

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Considerable evidence is accumulating concerning the role of growth factors in the metabolism of amino acids by microorganisms. Koser, Wright, and Dorfman (1942) demonstrated a relationship between aspartic acid and biotin in that aspartic acid can serve as a partial substitute for biotin in the growth of *Torula cremoris*. Pantothenic acid influences the synthesis of tryptophane by *Staphylococcus aureus* (Sevag and Green, 1944). A member of the vitamin B<sub>6</sub> group, pyridoxal phosphate, is the coenzyme for the decarboxylation of tyrosine, lysine, arginine, and other amino acids (Gale and Epps, 1944; Gunsalus, Bellamy, and Umbreit, 1944; Baddiley and Gale, 1945; Umbreit and Gunsalus, 1945). Pyridoxamine and pyridoxal are involved in the synthesis of lysine, threonine, and alanine by lactic acid bacteria (Stokes and Gunness, 1945). A combination of vitamin B<sub>6</sub> and CO<sub>2</sub> apparently promotes synthesis of arginine, phenylalanine, and tyrosine by *Lactobacillus arabinosus* (Lyman *et al.*, 1947).

Few data have been available concerning the specific role of biotin in the growth of microorganisms. That biotin must play an important metabolic role is indicated by the need for biotin by many microorganisms for growth, its wide distribution in cells, and its great activity per unit weight. The data presented below demonstrate that biotin is involved in the synthesis of aspartic acid by microorganisms. A preliminary report of this work has been published (Stokes, Larsen, and Gunness, 1947).

## METHODS

Stab cultures of the bacteria were carried in a medium of the following composition: 1 g of glucose, 0.5 g of Difco peptone, 0.6 g of anhydrous sodium acetate, salts A and B in half the concentration given in table 1, and 1.5 g of agar per 100 ml of medium, at pH 6.8. Inocula for the experiments were prepared by subculturing from stab cultures into a liquid medium of the same composition as that given above. After incubation for 16 to 24 hours at 37 C, the cells of the broth cultures were centrifuged, washed with water, and suspended in 100 ml of water. One drop of this suspension served to inoculate each tube in an experiment. The basal medium (table 1) was prepared as described previously (Stokes and Gunness, 1945) and distributed in 5-ml quantities in 22-by-150-mm tubes.

After addition of the experimental compounds, the volume in the tubes was brought to 10 ml with water prior to sterilization by autoclaving. Unless indicated otherwise, cultures of *Streptococcus faecalis* R were incubated for 40 hours and the remaining organisms for 64 hours at 37 C, at which times maximum

acid production has occurred. Lactic acid was determined by titration with alkali using bromthymol blue as the indicator. *S. faecalis* R cultures were titrated with .05 N NaOH and the other bacteria with 0.1 N NaOH. *S. faecalis* forms less acid than the lactobacilli in the basal medium employed. The titrations were made directly in the culture tubes. Growth is usually expressed in terms of the amount of acid formed in the cultures since the latter can be easily measured quantitatively. Synthetic *dl*-aspartic acid was used in all experiments. The biotin was *d*-biotin obtained from synthetic *dl*-biotin. Additional details of methods are described later.

TABLE 1  
*Basal medium*

<i>dl</i> -Leucine.....	100 mg	Sodium acetate (anhydrous).....	3 g
<i>dl</i> -Isoleucine.....	100 mg	Adenine.....	5 mg
<i>dl</i> -Valine.....	100 mg	Guanine.....	5 mg
<i>l</i> (-)-Cystine.....	100 mg	Uracil.....	5 mg
<i>dl</i> -Methionine.....	100 mg	Pantothenic acid.....	100 µg
<i>dl</i> -Tryptophane.....	200 mg	Riboflavin.....	100 µg
<i>l</i> (-)-Tyrosine.....	100 mg	Thiamine HCl.....	100 µg
<i>dl</i> -Phenylalanine.....	100 mg	Nicotinic acid.....	100 µg
<i>dl</i> -Glutamic acid.....	100 mg	Pyridoxamine.....	200 µg
<i>dl</i> -Threonine.....	100 mg	<i>p</i> -Aminobenzoic acid.....	20 µg
<i>dl</i> -Alanine.....	100 mg	Biotin.....	0.1 µg
<i>dl</i> -Aspartic acid.....	100 mg	Folic acid*.....	1.0 µg
<i>l</i> (+)-Lysine.....	50 mg	Salts A	
<i>l</i> (+)-Arginine.....	100 mg	K <sub>2</sub> HPO <sub>4</sub> .....	250 mg
<i>l</i> (+)-Histidine.....	100 mg	KH <sub>2</sub> PO <sub>4</sub> .....	250 mg
<i>dl</i> -Serine.....	100 mg	Salts B	
<i>l</i> (-)-Proline.....	100 mg	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	100 mg
<i>l</i> (-)-Hydroxyproline.....	100 mg	NaCl.....	5 mg
<i>dl</i> -Norleucine.....	100 mg	FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	5 mg
Glycine.....	100 mg	MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	5 mg
Glucose.....	5 g	Adjust to pH 6.8	
		Add distilled H <sub>2</sub> O to.....	250 cc

\* Obtainable from Dr. R. J. Williams, University of Texas, Austin, Texas; pteroyl glutamic acid may also be used.

Equivalent to 1.0 µg of material of "potency 40,000" or 1.0 µg of pteroyl glutamic acid.

#### EXPERIMENTS

In preliminary experiments designed to extend the basic microbiological assay method for the ten essential amino acids (Stokes, Gunness, Dwyer, and Caswell, 1945) to include the assay of aspartic acid, poor agreement of values at different levels of impure proteins was noted. The test organism was *Streptococcus faecalis* R, which in the usual synthetic media (table 1) requires aspartic acid for growth. An attempt was made to improve the basal medium by increasing the content of vitamins and the purine and pyrimidine bases fivefold. Surprisingly, this change caused almost maximum growth and lactic acid formation of *S.*

*faecalis* in the blank tubes which contained no aspartic acid. It appeared, therefore, that the increase in growth factor supplements stimulated synthesis of aspartic acid by *S. faecalis*. Fractionation of the growth factor mixture demonstrated that the increase in biotin alone was responsible for the growth of *S. faecalis* in the absence of aspartic acid (table 2). Increases in adenine, guanine, uracil, riboflavin, pantothenic acid, thiamine, nicotinic acid, *p*-aminobenzoic acid, pyridoxamine, and folic acid were ineffective in supporting appreciable growth in the absence of aspartic acid.

The ability of biotin to substitute for aspartic acid is not confined to *S. faecalis* R. A survey of eight additional aspartic-acid-requiring bacteria revealed that, with the exception of the heterofermentative *Leuconostoc mesenteroides* P-60,

TABLE 2  
Effect of increased concentrations of growth factors on development of  
*Streptococcus faecalis* R in the absence of aspartic acid

ADDENDUM*	GROWTH†	ML 0.05 N LACTIC ACID FORMED PER 10 ML OF MEDIUM†
Nil.....	+	2.1
Aspartic acid, 0.5 mg.,.....	++++	12.7
Fivefold increase in		
All vitamins + adenine, guanine, uracil.....	++++	11.4
Adenine, guanine, uracil.....	+	1.9
Riboflavin.....	+	2.2
Pantothenic acid.....	+	2.1
Thiamine.....	+	2.1
Nicotinic acid.....	+	2.0
Biotin.....	++++	10.2
<p>-Aminobenzoic acid.....</p>	+	2.1
Pyridoxamine.....	+	2.0
Folic acid.....	+	2.0

\* Added to the basal medium (table 1) from which aspartic acid was omitted.

† After incubation at 37 C for 40 hr.

addition of excess biotin to the basal medium resulted in full or almost full growth, as measured by acid production, of all strains of streptococci and lactobacilli tested in the absence of aspartic acid (table 3). For *Streptococcus faecalis* 10C1 and F24 and for *Streptococcus zymogenes* 5C1, 0.5 millimicrograms of biotin were sufficient to permit considerable growth in the absence of aspartic acid, although the stimulatory effect of additional biotin is clearly evident.

In figure 1 it can be seen that if a production of 6 ml of acid is used as a point of reference, it is necessary to supply the *Lactobacillus casei* strains with 3 to 5 times, and *L. arabinosus* with 2.7 times, as much biotin for growth in the absence of aspartic acid as when aspartic acid is present. Similar ratios were obtained for the other bacteria listed in table 3. It is also evident from the graph that the lactobacilli require biotin for growth even when liberally supplied with aspartic acid, a fact which indicates that biotin is required for metabolic func-

tions other than those concerned with synthesis of aspartic acid. From the quantitative biotin ratios given above, it appears that much more biotin is neces-

TABLE 3  
*Substitution of biotin for aspartic acid in the growth (acid formation)  
of various aspartic-acid-requiring bacteria*

MICROORGANISM	0.5 MILLIMICROGRAMS BIOTIN PER 10 ML MEDIUM		20 MILLIMICROGRAMS BIOTIN PER 10 ML MEDIUM	
	No aspartic acid	2 mg dl-aspartic acid	No aspartic acid	2 mg dl-aspartic acid
	ml acid formed per 100 ml medium*			
<i>Streptococcus faecalis</i> R.....	0.8	11.1	8.9	12.9
<i>Streptococcus faecalis</i> 10Cl.....	5.9	9.8	10.7	11.1
<i>Streptococcus faecalis</i> F24.....	4.7	9.8	11.0	12.1
<i>Streptococcus durans</i> 98A.....	3.1	11.1	10.8	11.5
<i>Streptococcus zymogenes</i> 5Cl.....	4.7	9.8	11.0	12.1
<i>Lactobacillus casei</i> LD5†.....	1.2	8.5	8.8	10.5
<i>Lactobacillus casei</i> .....	1.6	9.0	8.8	11.7
<i>Lactobacillus arabinosus</i> 17-5.....	0.7	9.2	11.0	11.5
<i>Leuconostoc mesenteroides</i> P-60.....	0.9	13.4	0.8	17.3

\* The lactobacillus cultures were titrated with 0.1 N NaOH and the remaining cultures with 0.05 N NaOH after 3 days' incubation at 37 C.

† Formerly known as *Lactobacillus delbrückii* LD5 but recently identified as a strain of *Lactobacillus casei* (Rogosa, 1946).

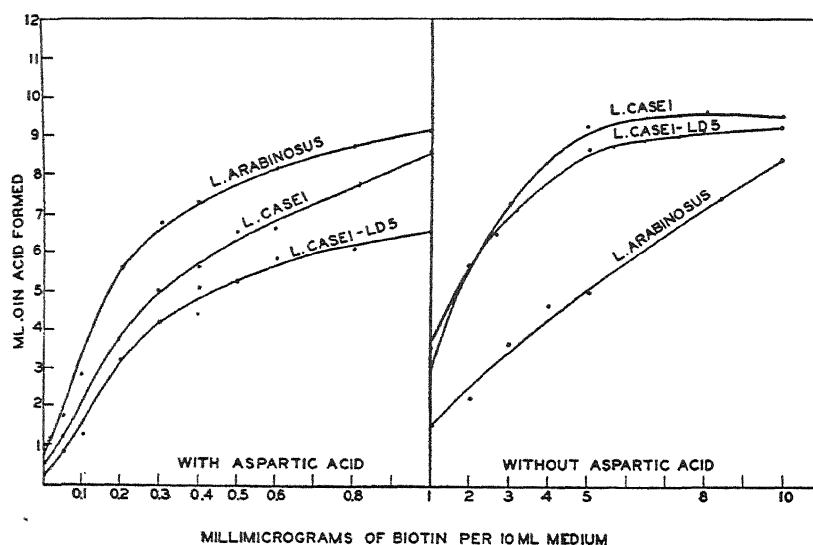


FIG. 1. QUANTITIES OF BIOTIN REQUIRED FOR GROWTH OF LACTOBACILLI WITH AND WITHOUT ASPARTIC ACID

sary for synthesis of aspartic acid than for the other function or functions of biotin. The need of the bacteria for biotin in the presence of aspartic acid elimi-



nates the possibility that all of the foregoing results could be explained by assuming that the bacteria do not require aspartic acid but that growth with aspartic acid is due to biotin present as an impurity in the aspartic acid.

The biotin-aspartic-acid relationship is very specific. As previously indicated (table 2) only biotin of the vitamins tested stimulated growth in the absence of aspartic acid. Also, although *S. faecalis* R, *L. arabinosus*, and *L. casei* require leucine, isoleucine, valine, cystine, methionine, tryptophane, tyrosine,

TABLE 4  
*Influence of biotin on the amino acid requirements of lactic acid bacteria*

AMINO ACID OMITTED	STREPTOCOCCUS FAECALIS R		LACTOBACILLUS ARABINOSUS 17-5		LACTOBACILLUS CASEI	
	0.0005 $\mu$ g Biotin	0.1 $\mu$ g Biotin	0.0005 $\mu$ g Biotin	0.1 $\mu$ g Biotin	0.0005 $\mu$ g Biotin	0.1 $\mu$ g Biotin
<i>ml acid formed per 10 ml medium</i>						
None.....	12.4	13.8	9.6	12.5	8.7	11.3
Leucine.....	0.8	1.2	0.6	0.6	0.5	0.6
Isoleucine.....	1.6	2.1	0.8	0.6	0.6	0.7
Valine.....	0.7	1.4	0.7	0.8	0.6	0.5
Cystine.....	9.2	11.3	6.3	8.9	0.7	0.7
Methionine.....	1.6	1.7	0.7	1.2	6.1	6.1
Tryptophane.....	1.1	1.6	0.6	0.8	0.7	0.7
Tyrosine.....	2.4	1.8	1.1	1.0	1.2	1.2
Phenylalanine.....	9.9	10.1	0.5	0.6	0.6	0.7
Glutamic acid.....	0.6	1.2	0.5	0.6	0.6	0.7
Threonine.....	0.6	0.7	8.8	9.8	8.6	9.7
Alanine.....	7.1	6.7	9.1	10.0	8.8	9.8
Aspartic acid.....	0.9	12.8	1.0	11.6	2.6	7.8
Lysine.....	1.0	0.8	8.8	10.2	8.5	9.6
Arginine.....	1.1	1.2	1.1	1.0	0.9	1.0
Histidine.....	1.6	1.7	8.4	9.6	8.3	8.8
Serine.....	7.0	6.9	8.3	9.4	0.4	0.6
Proline.....	12.3	13.1	8.8	10.6	8.6	9.0
Hydroxyproline.....	12.1	14.0	8.9	9.9	8.9	9.2
Norleucine.....	11.2	12.5	8.2	10.2	8.3	8.8
Glycine.....	7.9	9.8	7.7	8.1	7.8	8.2

phenylalanine, glutamic acid, threonine, lysine, arginine, histidine, and serine for growth in addition to aspartic acid, the requirement for only aspartic acid is eliminated by the use of excess biotin in the medium (table 4).

So far it has been assumed that the ability of biotin to substitute for aspartic acid in the nutrition of the bacteria indicates that biotin is involved in the synthesis of that amino acid. However, since it has been shown, apparently, that the proteins of certain algae are lacking in lysine, tyrosine, arginine, and methionine (Mazur and Clarke, 1938, 1942), it seemed necessary to prove that the bacterial cells grown with excess biotin in place of aspartic acid actually

contain aspartic acid. *S. faecalis* R, *L. arabinosus*, and *L. casei* LD5 were grown in the usual basal medium with 50 millimicrograms of biotin per 10 ml of medium and no aspartic acid, and also, as controls, in media containing 1 millimicrogram of biotin and 2 mg of *dl*-aspartic acid per 10 ml of medium. Each organism was grown in four 250-ml Erlenmeyer flasks, each containing 100 ml of medium. As customary, *S. faecalis* cultures were incubated for 2 days and the lactobacilli for 3 days at 37 C. As expected, controls consisting of media with 1 millimicrogram of biotin and no aspartic acid did not support growth. Macroscopically and microscopically there were no significant differences between cultures grown with and without aspartic acid. Also, the turbidity, acid production, and dry weight of the cell crops were determined. Fifty-milligram quantities of dry cells from each medium were hydrolyzed by autoclaving them for 5 hours at 15 pounds' pressure with 2 ml of 10 per cent HCl in sealed ampules.

TABLE 5

*Comparison of cultures grown with and without aspartic acid, with special reference to the aspartic acid content of the harvested cells*

DETERMINATION	S. FAECALIS R		L. ARABINOSUS		L. CASEI LD5	
	No aspartic acid	Aspartic acid	No aspartic acid	Aspartic acid	No aspartic acid	Aspartic acid
Turbidity.....	50*	42	15	22	18	20
Ml acid formed.....	10.0	12.4	9.6	10.3	9.1	11.4
Mg dry cells.....	99	120	201	97	212	150
% aspartic acid in cells.....	5.5	5.4	6.1	6.2	4.9	5.0

\* Per cent transmissible light (Evelyn colorimeter); uninoculated medium = 100.

The hydrolyzates were neutralized, filtered, diluted to 50-ml volume, and assayed for aspartic acid content with *Leuconostoc mesenteroides* (Hac and Snell, 1945). It will be recalled that for *L. mesenteroides*, biotin cannot substitute for aspartic acid. As shown in table 5, the bacterial cultures with and without aspartic acid are quite similar in turbidity, acid production, crop yield, and aspartic acid content of the harvested cells. There is no doubt that aspartic acid is synthesized, and in normal quantities, by the bacterial cells grown without aspartic acid in the presence of excess biotin. It seems reasonable to conclude, therefore, that biotin is involved in the synthesis of aspartic acid by those microorganisms.

The sulfur-free derivative of biotin, *d*-desthiobiotin, and the biotin stereoisomers, *dl*-allobiotin and *dl*-epiallobiotin (Stokes and Gunness, 1945), failed to support growth of *S. faecalis*, *L. casei*, *L. casei* LD5, and *L. arabinosus* when used in place of biotin in aspartic-acid-free media. The oxygen analog of biotin *dl*-*o*-heterobiotin<sup>1</sup> (Duschinsky *et al.*, 1945) is an effective substitute for biotin for *S. faecalis* and *L. arabinosus*, but not for the *L. casei* strains. However, 100 to 500 times as much *o*-heterobiotin as biotin must be used. The diamino-

<sup>1</sup> Kindly supplied by Dr. Saul Rubin of Hoffman-La Roche, Inc.

carboxylic acid derivative of biotin obtained synthetically as *dl*-diamino acid sulfate (Stokes and Gunness, 1945) can completely replace biotin in aspartic-acid-free media for the four organisms mentioned above but 10,000 times as much of this compound as compared to biotin must be used for full growth.

The ability of *S. faecalis* R to grow without aspartic acid in the presence of excess biotin appears to be a characteristic of at least the majority of the cells in the parent culture, since 22 single colony isolates obtained by plating all showed the same phenomenon. Also, the four organisms listed above developed fully through seven serial loop subcultures in the basal aspartic-acid-free medium containing 20 millimicrograms of biotin, a result which indicates that the biotin-aspartic-acid phenomenon is independent of any factor that might have been carried over from the original peptone glucose sodium-acetate inoculum medium. The ability of biotin to substitute for aspartic acid is independent of the pH of the medium between pH 6 to pH 8; at pH 5 growth is submaximum even when aspartic acid is supplied to the medium. Varying the pH of the growth medium between pH 5 and pH 8 did not alter the need of *Leuconostoc mesenteroides* for aspartic acid, although excess biotin, 20 millimicrograms per 10 ml, was in the medium. Also, almost invariably initiation of growth of the bacteria in aspartic-acid-free medium lags by 10 to 20 hours behind simultaneously inoculated media containing aspartic acid. Apparently, forcing the organisms to synthesize their required quota of aspartic acid greatly increases the lag phase.

Although biotin can replace aspartic acid in the nutrition of many bacteria that under some conditions at least appear to require both compounds for growth, the reverse is not true. *Saccharomyces cerevisiae* F6.4 (Snell, Eakin, and Williams, 1940) and *Neurospora sitophila* (Stokes, Larsen, Woodward, and Foster, 1943) both require biotin for growth. With the media and procedures described in the literature cited, aspartic acid at a concentration of 2 mg of the *dl* form per 10 ml of medium failed to eliminate the need of the yeast and the mold for biotin. This inability of aspartic acid to substitute for biotin is understandable in view of the results given above, which demonstrate that biotin is also required by the bacterial cells for metabolic functions other than those concerned with the synthesis of aspartic acid. The multiple function of biotin probably also explains the inability of Koser *et al.* (1942) completely to replace biotin by aspartic acid in the case of *Torula cremoris*.

#### *Experiments with Resting Cell Suspensions*

A number of experiments were made with resting cell suspensions to determine the specific aspartic-acid-forming reaction catalyzed by biotin. The following reactions which are known to lead to or might be expected to give rise to aspartic acid in biological systems were investigated:

1. Glutamic acid + oxalacetic acid  $\rightarrow$  aspartic acid +  $\alpha$ -keto-glutaric acid (Cohen and Hekhius, 1941).
2. Alanine + oxalacetic acid  $\rightarrow$  aspartic acid + pyruvic acid (Cohen and Hekhius, 1941).

3. Cysteic acid + oxalacetic acid  $\rightarrow$  aspartic acid + sulfapyruvic acid (Cohen and Hekhius, 1941).
4. Succinic acid  $\xrightarrow{-2H}$  fumaric acid  $\xrightarrow{+H_2O}$  malic acid  $\xrightarrow{-2H}$  oxalacetic acid (Harrow, 1940); followed by reaction (1) to give aspartic acid.
5. Fumaric acid +  $NH_3 \rightarrow$  aspartic acid (Quastel and Woolf, 1926).
6. Pyruvic acid +  $CO_2 \rightarrow$  oxalacetic acid (Krampitz, Wood, and Werkman, 1943); followed by reaction (1) to give aspartic acid.

It is evident that with the exception of reaction (5), transamination is directly or indirectly involved in all of them.

In a typical experiment *L. arabinosus* was grown in 250-ml Erlenmeyer flasks containing 100 ml of the basal medium (table 1). The biotin content, however, was reduced to the very small quantity of .05 millimicrogram per 10 ml of medium, and 200  $\mu$ g of oleic acid per 10 ml were added as a substitute for the remainder of the required biotin (Williams and Fieger, 1946). The medium was adjusted to pH 5.6. Cells harvested from such a medium are essentially free from biotin. After incubation for 3 days at 37 C, the cells were collected by centrifugation, washed once with M/15 phosphate buffer at pH 7, and resuspended in sufficient buffer of the same type to give a galvanometer deflection of 5 on the Evelyn photoelectric colorimeter at 520 millimicrons wave length. Four-ml aliquots of cell suspension were mixed in test tubes (22 by 150 mm) with 10 mg each of the compounds shown in table 6 except that 20 mg of *dl*-alanine were used. To one of duplicate sets, 5  $\mu$ g of biotin were added. Where necessary, the pH of the suspensions was adjusted to pH 7 and the volume to 5 ml. The thoroughly shaken tubes were stoppered and incubated overnight, for approximately 18 hours at 37 C. After incubation, the aspartic acid in the suspensions, cells plus fluid, was determined by quantitative assay with *Leuconostoc mesenteroides* (Hac and Snell, 1945) employing the medium shown in table 1 and a total assay volume of 1.0 ml. Titrations were made with 0.01 N NaOH. In this way as little as 2  $\mu$ g to 10  $\mu$ g of aspartic acid per ml of suspension could be readily measured (figure 2).

It is evident from table 6 that resting cells of *L. arabinosus* form aspartic acid from glutamic acid, alanine, or cysteic acid plus oxalacetic acid. Malic and fumaric acids and to a lesser extent succinic acid can substitute for oxalacetic acid, presumably because they are converted to oxalacetic acid by the resting cells. However, all of these reactions proceed as well without biotin as with it. This clearly indicates that biotin is not involved in any of these reactions. The cells in this particular experiment contained less than .04 millimicrograms of biotin per ml of suspension as measured microbiologically (Wright and Skeggs, 1944) on acid-hydrolyzed cells. Therefore, the possibility of carry-over of significant amounts of biotin by the cells is eliminated. Glutamine can replace glutamic acid to give aspartic acid with either malic, fumaric, or succinic acid. Similar results were obtained with *L. arabinosus* cells grown in media containing excess biotin and no aspartic acid, and also in media with vitamin-free casein hydrolyzate, as a substitute for all of the amino acids except cystine and trypto-

phane, plus either oleic acid or sufficient biotin for half-maximum growth (0.2 millimicrograms per 10 ml) to reduce carry-over of biotin from the medium by the cells. In general, resting cells of *S. faecalis* R and *L. casei* gave results similar

TABLE 6  
*Formation of aspartic acid by cell suspensions of Lactobacillus arabinosus*

CELLS PLUS ADDENDA	NO BIOTIN	PLUS BIOTIN
	Aspartic acid	
	micrograms per ml of suspension	
Nil.....	3	3
Glutamic acid + oxalacetic acid*.....	28	26
Glutamic acid + malic acid.....	108	104
Glutamic acid + fumaric acid.....	140	148
Glutamic acid + succinic acid.....	14	14
Alanine + oxalacetic acid.....	14	14
Cysteic acid + oxalacetic acid.....	13	17

\* Ninety-two per cent pure; kindly supplied by Dr. L. O. Krampitz.

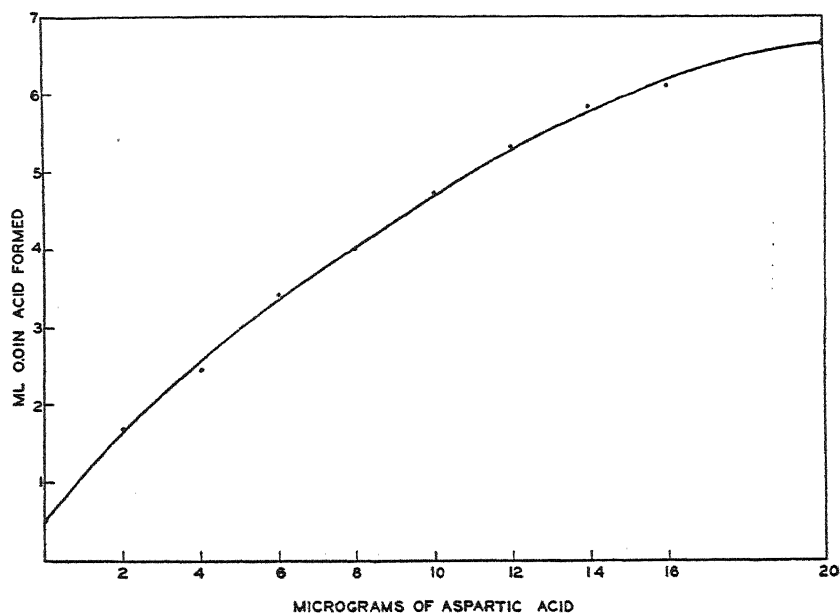


FIG. 2. RESPONSE OF *LEUCONOSTOC MESENTEROIDES* TO *L*-ASPARTIC ACID

to those obtained with *L. arabinosus*. Indirect evidence that biotin does not catalyze any of the reactions in table 6 is the fact that resting cells of *L. mesenteroides*, whose requirement for aspartic acid is not influenced by biotin, also produce aspartic acid under these conditions.

No evidence could be obtained with *L. arabinosus* for the formation of aspartic acid by the direct amination of fumaric acid. Similarly negative results were obtained with malic or succinic acid and  $(\text{NH}_4)_2\text{SO}_4$ . In this connection, however, it may be significant that biotin-deficient yeast cells are markedly stimulated by biotin to take up ammonia (Winzler, Burk, and duVigneaud, 1944). Our negative results may merely indicate that the proper physiological conditions were not provided in the resting cell suspension experiments.

Also, no aspartic acid was formed in cell suspensions of *L. arabinosus* supplied with glutamic acid, plus pyruvic acid and either  $\text{NaHCO}_3$  or  $\text{CO}_2$  gas as a source of carbon dioxide. These negative results were not altered by the addition of thiamine, pyridoxamine, *p*-aminobenzoic acid, riboflavin, pantothenic acid, nicotinic acid, folic acid, glucose, and adenosine triphosphate to the suspensions;

TABLE 7  
Stimulation of growth (acid formation) of lactic acid bacteria  
by oxalacetic acid in aspartic-acid-free medium

COMPOUND ADDED	<i>L. CASEI</i>	<i>S. FAECALIS</i>	<i>L. ARABINOSUS</i>	<i>L. MEESENTEROIDES</i>
Per 10 ml medium*	ml 0.1 N acid formed per 10 ml medium			
Nil.....	4.6	0.8	3.3	0.5
<i>dl</i> -Aspartic acid, 2 mg.....	9.6	6.8	9.5	8.7
Biotin, 0.1 $\mu\text{g}$ .....	9.6	6.1	10.0	0.5
Oxalacetic acid, † 1 mg.....	7.9	0.8	4.1	0.5
Oxalacetic acid, 5 mg.....	10.0	0.8	6.3	0.5
Oxalacetic acid, 25 mg.....	9.4	0.7	7.5	0.5

\* Basal medium contained 0.8 millimicrograms of biotin and no aspartic acid.

† Sterilized by filtration.

by varying the pH of the suspension from pH 6 to pH 8; nor by the use of acetone-dried cells possibly to increase permeability of the cells to adenosine triphosphate. The acetone-dried cells readily formed aspartic acid when mixed with glutamic and oxalacetic acids.

A suggestion that biotin may be concerned with the formation of oxalacetate was obtained from growth experiments in which for *L. casei* and *L. arabinosus* but not for *S. faecalis* R oxalacetic acid partially replaced biotin in aspartic-acid-deficient media (table 7). The possibility that the activity of the oxalacetic acid was due to impurities of biotin or aspartic acid was ruled out by assay of the preparation for these two components.

#### SUMMARY

Biotin can completely substitute for aspartic acid in the growth of *Lactobacillus arabinosus*, *Streptococcus faecalis*, and related organisms. The biotin-aspartic-acid relationship is specific; riboflavin, pantothenic acid, thiamine, *p*-aminobenzoic acid, and pyridoxamine cannot replace biotin, nor can biotin substitute for 14 amino acids other than aspartic acid which are required for growth. Cells grown with biotin contain as much aspartic acid as those grown

with aspartic acid. It is concluded that biotin participates in the synthesis of aspartic acid. Although resting cell suspensions of *Lactobacillus arabinosus* can form aspartic acid by typical transamination reactions, the presence of biotin is not required for such reactions. It has not been possible to determine the specific aspartic-acid-forming reaction catalyzed by biotin.

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# THE MORPHOLOGY OF THE L<sub>1</sub> OF KLIENEBERGER AND ITS RELATIONSHIP TO STREPTO- BACILLUS MONILIFORMIS<sup>1</sup>

L. DIENES

*Department of Pathology and Bacteriology of the Massachusetts General Hospital  
and the Robert W. Lovett Memorial of Harvard Medical School, Boston,  
Massachusetts*

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In 1945 the author published observations concerning the morphology of the pleuropneumonia group of organisms (Dienes, 1945). The methods used for the study of these organisms have been applied in the present work to *Streptobacillus moniliformis* and its L<sub>1</sub> variant. These organisms have been studied previously (Dienes, 1942), but improvements of technique in the meantime have made it possible to observe more clearly the form of the individual organisms and their derivation from each other. The properties of the L<sub>1</sub> and its connection with the bacillus challenge several accepted concepts in bacteriology. Most authors, taking into consideration that the L<sub>1</sub> originates from the bacillus, that it is serologically similar, and that under appropriate conditions it reproduces the bacillus, accept the conclusion that these two organisms, so different in appearance, are growth forms of the same organism. Klieneberger (1942) recently reaffirmed her objections to this conclusion; her chief objection is that the morphology of the L<sub>1</sub> is different from that of bacteria. Hence more accurate information should bring into agreement the different views on its nature. Such information is needed also as a basis to establish the biological significance of these peculiar bacterial forms.

The difficulties which prevented for a long time an adequate concept of the morphology of the pleuropneumonia group of organisms are present in L<sub>1</sub> to an even greater extent. The organisms are exceedingly fragile and soft; they adhere firmly to each other; and the colonies grow into the agar. In broth, soft dense clumps are formed. The best way to overcome these difficulties, as in the case of the pleuropneumonia group, has been in the staining of the colonies on the agar. By studying several strains in various stages of development it was possible to observe the forms which comprise the colonies. The agar fixation method (Klieneberger and Smiles, 1942), which gives excellent preparations with many strains of the pleuropneumonia group, is not applicable to small L<sub>1</sub> colonies, because they either do not adhere to the glass or, if they adhere, they are in dense clumps. Only the surface of well-developed colonies adheres to the glass, and some of the most successful photographs, both of the small and large forms of the organisms, were made from such preparations. This method alone is not, however, sufficient for the study of the cultures because it does not allow one to

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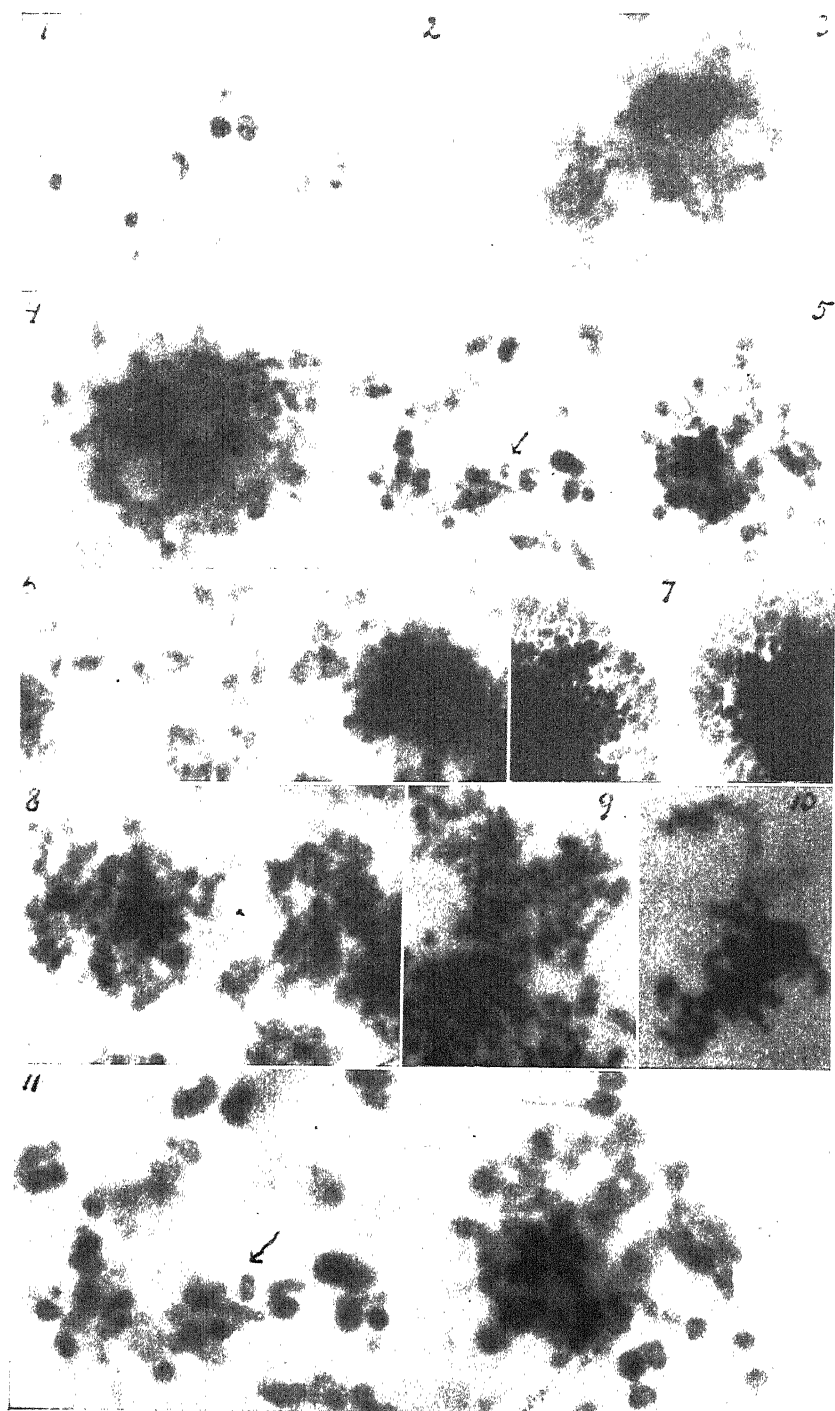


FIG. 1

observe the whole course of their development. The following studies are based on three strains of *Streptobacillus moniliformis* and the  $L_1$  variants derived from them.

The organisms in  $L_1$  colonies appear in two main forms with intermediate transitional forms. The young colonies and the central mass of well-developed colonies consist of small forms. These are transformed at the surface and the periphery of the colonies by gradual swelling into large round forms similar in every respect to the large forms present in colonies of the pleuropneumonia group of organisms and of various bacteria.

The shape of the small organisms appeared most clearly in preparations made from cultures on coagulated egg. The colonies grew on the surface of this medium without penetrating it. Impression preparations were made from the cultures following agar fixation. As mentioned above, only the surface of the colonies adheres to the glass, but in some preparations a few small forms were mixed with the large bodies usually present on the surface. The smallest organisms appeared to be distinctly bacillary. Photograph no. 2 of figure 1 made from such a preparation shows a tiny bipolar-stained bacillus. In photograph no. 1 of figure 1 three small organisms adhere to each other forming a small filament. Although they are darker stained than the organism in the following photograph, their shape is distinctly bacillary. A slightly swollen organism in the same photograph shows polar staining, which is more or less apparent in some of the larger forms.

In wet stained agar preparations the appearance of the small organisms was similar. The youngest colonies consist of small bacillary forms sometime showing bipolar staining. Vigorously growing young colonies were obtained with the following procedure. The fresh medium was covered with an agar square cut from a 24-hour culture and incubated for 8 to 13 hours. The old agar square was discarded and the agar under it containing a fresh growth was studied. The organisms in photographs no. 5 and 11 of figure 1 showed the bacillary form clearly and, in some cases, bipolar staining as well. It is hoped that their shape

#### FIGURE 1

No. 1. Impression preparation from a young  $L_1$  colony grown on coagulated egg. Fixation through the medium with Bouin's solution. Staining with methylene blue and azur.  $\times 3,000$ . Individual organisms are clearly visible, a short bacillary filament consisting of 3 bacilli, a bipolar-stained short bacillus, and consecutive stages in the development of large round forms. In one round form the polar staining remains visible.

No. 2. A bipolar-stained bacillus from the same preparation as no. 1.  $\times 3,000$ .

Nos. 3 and 4. The surface of small  $L_1$  colonies photographed from wet stained agar preparation.  $\times 3,000$ . In the lower part of no. 3, a few bacillary forms are discernible besides moderately swollen forms. In no. 4, the colony consists of small forms whose exact shape is not clearly visible.

Nos. 5, 6, and 11. Stained wet agar preparations. In no. 6 ( $\times 3,000$ ), small bacillary forms and one round body are visible. In no. 5 ( $\times 3,000$ ), many bacillary forms are visible, usually arranged in small clumps. One small bacillus marked with an arrow shows bipolar staining. No. 11 is the same as no. 5 enlarged to  $\times 4,500$ . The shape of the organisms is only occasionally apparent, because they are parts of small clumps most of which, of necessity, are out of focus.

No. 7. Well-developed colonies with moderate magnification. Stained dry agar preparation.  $\times 500$ . The dense center consists of small forms embedded in the agar; the periphery consists of large round bodies situated on the surface of the agar.

Nos. 8, 9, and 10. Small  $L_1$  colonies photographed from dried stained agar preparations.  $\times 3,000$ . The shape of individual organisms is not clearly visible, but it is apparent that their arrangement is similar to that of bacteria in bacterial colonies.

and structure will remain visible in the reproductions. Although the fact that the organisms are not in one plane and that they adhere in clumps makes it difficult to obtain sufficiently clear photographs.

Photographs no. 8, 9, and 10 were made from preparations similar to the preceding ones with the difference that the thin agar slices were dried on the cover slips. Drying compresses the cultures vertically, and for this reason a large part of the colony is seen in sharp focus. On the other hand, the individual organisms are not so distinct as in wet preparations. The most important informations obtained from the study of dry agar preparations is that the arrangement of the organisms in the youngest colonies is similar to the arrangement of bacteria in bacterial colonies, an observation indicating a similarity of growth and reproduction. The small bacillary forms in the photograph made from both wet and dry preparations are about  $\frac{2}{3}$  of a micron long and  $\frac{1}{3}$  wide.

The small forms in the  $L_1$  colonies are transformed by gradual swelling into large bodies. In photographs no. 1 and 6 of figure 1 the consecutive stages of this process are apparent. When the large bodies are fully developed, they sometimes appear to be filled with small bacillary forms similar to those growing in young colonies. These small forms are tightly packed in several layers, and their actual forms can be seen only occasionally. They must be soft and fragile, because even slight mechanical injury to the large body destroys them. The structure of the large bodies was most clearly seen in preparations stained with safranine after fixation with Bouin's solution through the agar. Photographs 1 and 4 of figure 2 were made from such preparations.

The development of the large bodies into  $L_1$  colonies in transplants has been previously described. They increase in size, their contours may become uneven, and the small  $L_1$  organisms grow out of them at one or at several points. The large body does not germinate as a single organism, like a yeast cell, for example, but it apparently contains numerous small organisms capable of growth. The author agrees in this point with Klieneberger (1942), but he has seen no indication of the process, postulated by Klieneberger, by which previously separate organisms develop a common membrane and form a large body. The gradual swelling of the small forms into large bodies is apparent in the cultures.

The development of  $L_1$  has been followed on thin agar slices under a cover slip and in stained agar preparations made from the cultures at short intervals. No other viable organisms were discernible in the cultures except the small bacillary forms, the large bodies, and the intermediary forms. The viable organisms are stained intensely blue by methylene blue, and the cultures stop developing in transplants when the blue staining disappears from the colonies. Autolysis produced granules of various size which are stained pink both by methylene blue and Giemsa solution. These granules never show multiplication in transplants. Similar granules are produced in autolyzed cultures of *Streptobacillus moniliformis* and are regarded by Klieneberger as forms of  $L_1$  (1942). According to the experience of the author these granules do not multiply and there is no reason to believe that they represent the  $L_1$ . When a *Streptobacillus* culture which is not grossly mixed with  $L_1$  colonies is transplanted, the  $L_1$  develops exclusively from large bodies produced by swelling of the bacilli.

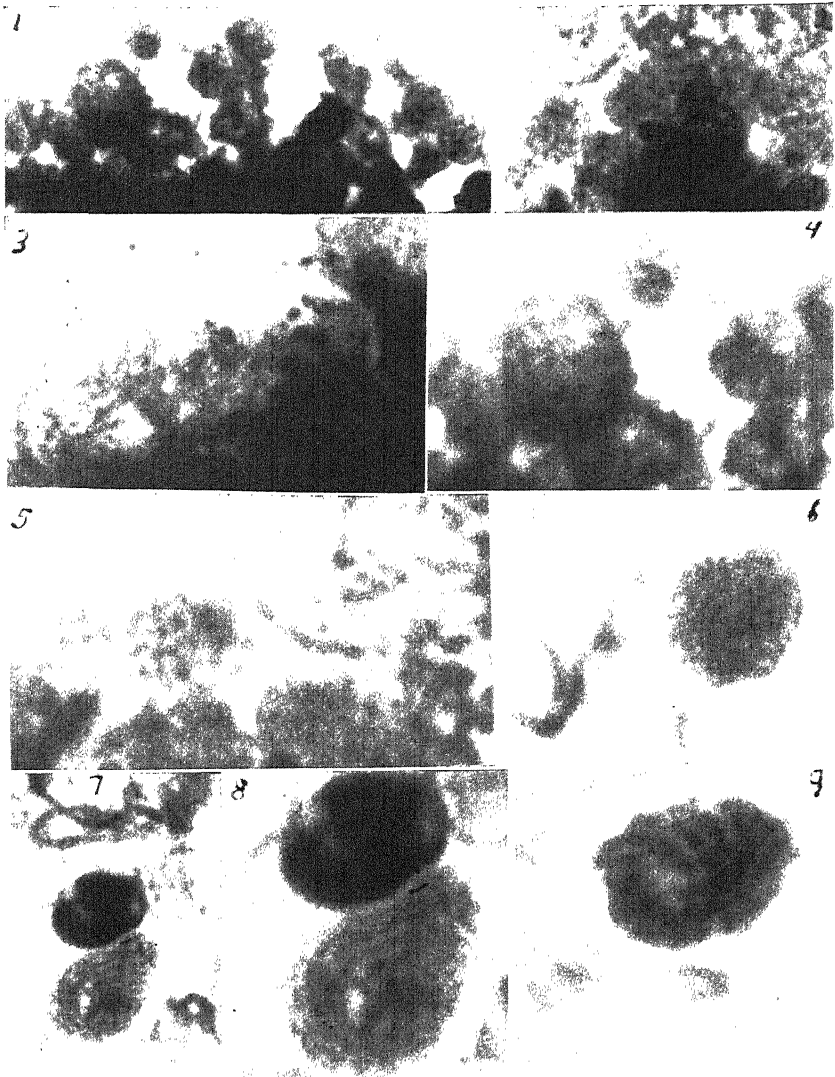


FIGURE 2

No. 1. Safranine staining after agar fixation.  $\times 3,000$ . Darkly stained small bacillary forms surrounded by halos are apparent in several large bodies.

No. 2. Large bodies filled with small bacillary forms in a culture of *Streptobacillus moniliformis*. Safranine after agar fixation.  $\times 3,000$ .

No. 3. A large body in a culture of *Streptobacillus moniliformis* filled with round granules. Methylene blue and azur after agar fixation.  $\times 3,000$ .

No. 4 and 5. Enlargement of nos. 1 and 2, respectively, to  $\times 4,500$ .

No. 6. A large body in a *Streptobacillus* culture filled with bacilli of the usual shape. Giemsa staining after agar fixation  $\times 3,000$ .

No. 7. Large body in a *Streptobacillus* culture filled with bacilli of the usual shape. Giemsa staining after agar fixation.  $\times 3,000$ .

No. 8. Same as no. 7, enlarged to  $\times 4,500$ .

No. 9. A large body similar to those in nos. 6 and 7 stained with Victoria blue.  $\times 4,500$ .

The development of  $L_1$  in the cultures of *Streptobacillus moniliformis* from the the bacteria has been previously described (Dienes, 1942). The bacilli swell first into large fusiform bodies and under appropriate conditions these develop into  $L_1$  colonies. These large bodies are in appearance and physical properties similar to the large bodies of  $L_1$ . When they are fully developed, they are filled with similar soft bacillary forms and they develop into  $L_1$  colonies in a similar way. Transformation of the usual bacilli into  $L_1$  occurs during the development of the large body. Photographs no. 2 and 4 of figure 2 show large bodies developing in cultures of *Streptobacillus moniliformis* filled with small bacillary forms. In photograph no. 3 the small bodies developed into round forms, and it is clearly apparent that the large body contains many individual organisms.

Although most of the large bodies develop as indicated above, in certain cultures some large bodies develop in a different way. These large bodies, a few hours after transplantation, appear to be filled with bacilli of the usual shape and develop into regular bacillary colonies. Their growth produces first a tiny dense round colony very different from the usual growth of *Streptobacillus*; after a few hours they lose this character and become similar to the other bacterial colonies. The development of the large bodies into bacteria was described in a former paper (Dienes, 1943). The illustrations in this paper were not successfully reproduced and are replaced here by better ones. Attention is again called to the fact that every strain of *Streptobacillus* and the  $L_1$  isolated from it present marked individual properties. It is often impossible to observe in a given strain phenomena easily seen in others.

#### DISCUSSION

The observations described give further support to the view that the morphology of the  $L_1$  is bacterial. The small organisms in  $L_1$  colonies are tiny, often bipolar-stained bacilli. Similar forms are visible inside the large bodies developing either in bacterial or  $L_1$  cultures. These small bacillary forms share with the parent organism the tendency to swell to round forms. The  $L_1$  is more pleomorphic and has a more pronounced tendency to autolysis than the parent organism, but it is essentially similar to the parent organism both in regard to form and to reproduction. The morphological differences between the  $L_1$  and the parent organism were exaggerated by the use of inappropriate methods of observation. The differences between them are actually not more pronounced than those between a smooth and a very rough pneumococcus colony. It was mentioned above that large bodies found in the cultures of *Streptobacillus moniliformis* can develop either into  $L_1$  or into usual bacillary forms. This together with the observation that the  $L_1$  for a certain period after isolation returns easily into the *Streptobacillus* indicates that the  $L_1$  is apparently an intermediary link in the reproduction of the usual bacilli from the large bodies.

All these observations are in agreement with the supposition that the usual bacillary forms and the  $L_1$  are growth forms of the same organism. The only characteristic in the development of  $L_1$  which was previously not noticed in bacterial variation is that the change into  $L_1$  is preceded by a morphological change

of the parent organism, by swelling into large round forms. These processes are not exceptional in *Streptobacillus moniliformis*, but they are widely distributed in gram-negative bacilli (Dienes, 1942, 1946). To all appearances these processes represent a complex reproductive process different from binary fission. The author has pointed out that the  $L_1$  shows many similarities to the so-called "haploform" yeast of Winge (Dienes, 1946).

The morphology of the  $L_1$  is similar in all essential characteristics to the pleuropneumonia group of organisms. Without knowing the origin of a culture after it has lost its ability to return into the *Streptobacillus*, it would be impossible to recognize its identity on the basis of morphology.

#### SUMMARY

It is apparent in appropriate preparations that the small forms of the  $L_1$  colonies are small, often bipolar-stained bacilli. They enlarge by gradual swelling into large forms in which the small bacillary forms are again reproduced. The large bodies produced by swelling of bacteria in cultures of *Streptobacillus moniliformis* contain similar small bacillary forms, and when they germinate they produce an  $L_1$  colony. Sometimes the large bodies in the *Streptobacillus* culture are filled with bacilli of the usual shape and reproduce the usual bacillary colonies. The morphology of the  $L_1$ , like that of the whole pleuropneumonia group, is bacillary, and the swelling into large round forms and reproduction by these large forms is similar in nature to the analogous processes observed in other bacteria.

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# PRODUCTS OF ANAEROBIC GLYCEROL FERMENTATION BY STREPTOCOCCI FAECALIS

I. C. GUNSALUS

*Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York*

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The fermentation of oxidized or reduced substrates by homofermentative lactic acid bacteria must necessarily lead either to products other than lactic acid, or must require external hydrogen donors or acceptors. In a study of the fermentation of glycerol, a reduced substrate, by streptococci Gunsalus and Sherman (1942) noted among the enterococci two types of behavior; certain strains fermented glycerol readily with a limiting pH about 5, whereas others fermented the substrate slowly and reached a final pH of 5.5 to 6. The latter were found to require oxygen in order to utilize glycerol as an energy source. The glycerol metabolism of a strain of this type has been studied in some detail (Gunsalus and Umbreit, 1945).

In connection with the anaerobic fermentation of glycerol, Braak (1928) found with colon-aerogenes organisms that growth would cease before the glycerol was exhausted and would start again if more peptone or yeast extract was added. It seemed not unlikely that such a phenomena might also occur with lactic acid organisms.

The present paper deals with a strain of *Streptococcus faecalis* that ferments glycerol readily, with good growth, under anaerobic conditions. With this strain, yeast extract in addition to glycerol is needed for anaerobic growth, whereas with glucose as substrate yeast extract is not required. The yeast extract can be replaced with fumaric acid if a sufficient level of riboflavin is present—the fermentation products being primarily lactic and succinic acids.

## METHODS

*Culture.* *Streptococcus faecalis*, strain 10C1, a typical enterococcus from the departmental culture collection, has been used throughout these studies. This strain, and others which ferment glycerol anaerobically, grows more abundantly in ordinary laboratory media than strains which ferment glycerol only aerobically. These strains also attack a wider range of substrates and yield a wider variety of products (Gunsalus and Campbell, 1944; Gunsalus and Niven, 1942).

*Growth and media.* The growth was measured turbidimetrically as described previously in papers from this laboratory (Gunsalus and Sherman, 1942). Anaerobic conditions were obtained either by vaspar seals or the chromium-sulfuric acid method as described by Mueller and Miller (1941). The turbidity was measured at suitable intervals, and the final pH was determined at the end of the experiments, with a Beckman pH meter.

## RESULTS

In order to estimate the amount of growth supported by glycerol as substrate, a comparison was made of the growth in the base medium, and in this medium with glycerol and with glucose as substrates. In these studies extra buffer was avoided in order that the influence of pH would not be further masked if slight fermentation of glycerol occurred.

*Influence of Yeast Extract and Oxygen*

Since glycerol is more reduced than lactic acid, and since oxygen acts as an aerobic hydrogen acceptor in glycerol fermentation, it was considered possible that anaerobically some constituent of the medium might serve as a hydrogen acceptor. To test the effect of media constituents on growth and fermentation,

TABLE 1

*Effect of yeast extract and oxygen upon growth on glycerol and glucose*

*Streptococcus faecalis* 10C1

Base Medium: 1 per cent tryptone

Incubation: 10 days, 37 C (anaerobic series in chromium-sulfuric-acid jar)

YEAST EXTRACT	AEROBIC GROWTH*			ANAEROBIC GROWTH		
	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%						
0	14	21	125	12	14	125
0.2	19	52	170	19	45	160
0.5	28	70	200	30	60	170
1.0	47	115	210	44	74	190

\* Turbidity 1 scale unit  $\cong$  6  $\mu$ g bacterial N/10 ml.

the yeast extract level was altered as shown in table 1. One per cent tryptone supports slight growth of *Streptococcus faecalis*, strain 10C1, and glycerol improves the growth a little, whereas the addition of glucose as an energy source results in abundant growth. Not only is the anaerobic growth with glycerol poor, but aerobic growth is also slight. Therefore the tryptone must be deficient in factors necessary for the hydrogen transport to oxygen; otherwise, aerobic glycerol fermentation should occur (Gunsalus and Sherman, 1942). In the base medium yeast extract improves the growth slightly; and the further addition of glycerol provides moderate growth stimulation. The presence of oxygen affords some stimulation beyond that due to the presence of yeast extract, indicating that the quantity of hydrogen acceptor might be limiting. On the other hand, the final pH (5.0 with glycerol) may become limiting before maximum growth is attained. The growth, final pH, and titratable acidity for several variations in the medium are shown in table 2. Although very little acid was formed in the base medium, the final pH with yeast extract alone fell to 6.0. In the presence of glycerol the limiting pH was reached in all media containing 0.5 per cent, or more, yeast extract regardless of the level of tryptone. The glycerol supported about one-

third the growth, and about one-third the acid production, afforded by glucose. Whether this is due to the difference in fermentation pattern, or more likely, to the difference in growth afforded by the higher limiting pH with glycerol, cannot be determined by these data. The growth with glycerol as substrate can be about doubled by the addition of 0.5 per cent dipotassium phosphate; the limiting pH is not always reached in this case. Glycerol will support growth and acid production only when the yeast extract is added to the medium.

*Fractionation of Yeast Extract and Replacement with Fumaric Acid  
and Riboflavin*

Initial attempts to fractionate the yeast extract, as by ether extraction, resulted in two fractions neither of which was markedly active alone. However, on recombination they exhibited the full activity of yeast extract. Some fractions caused lower pH without much stimulation of growth, whereas others stimulated

TABLE 2  
*Anaerobic glycerol fermentation*  
*Streptococcus faecalis* 10Cl

Incubation: 8 days, 37 C in anaerobic jar

TRYPTONE	YEAST EXTRACT	GROWTH*			FINAL pH			ACID PRODUCTION		
		Base	Glycerol	Glucose	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%	%							<i>ml N/10 acid/10 ml</i>		
1	0	16	19	88	6.9	6.4	3.9	.1	0	3.6
1	0.5	18	56	90	6.6	5.2	4.0	0	1.1	3.9
1	1	22	58	110	6.7	5.1	4.1	0	1.6	4.6
0.5	1	18	44	74	6.6	5.0	4.1	.4	1.0	4.8
0	1	10	24	53	6.0	4.9	4.0	.4	.4	4.4

\* Turbidity 1 scale unit  $\cong$   $\mu$ g bacterial N.

growth without as great a depression in pH, thus suggesting the possibility that more than one substance was involved.

Therefore, several attempts were made to replace the yeast activity by known compounds. As shown in table 3, with glycerol as substrate in a tryptone medium, a small amount of yeast extract will stimulate growth only slightly but will stimulate more rapid acid production—final pH 5.0. The acid production is also stimulated by a mixture of accessory factors, or by riboflavin. In the presence of traces of yeast extract, or accessory factors, fumaric acid will greatly stimulate the growth. Although fumaric acid can replace the yeast extract, it does not necessarily follow that the action of yeast extract is due to fumaric acid.

Presumably, the fumarate acts as a hydrogen acceptor and additional riboflavin is needed for hydrogen transport. The data in table 4, with a synthetic medium, show plainly the increased riboflavin requirement. With the riboflavin and fumarate, glycerol fermentation proceeds rapidly, 12-hour growths being recorded, and appears not to require further factors beyond the requirement for growth in glucose. It should be noted, however, that the growth with glycerol as substrate still does not equal that with glucose.

TABLE 3  
Factors affecting glycerol fermentation

Incubation: 2 days, 37 C Medium: 1% tryptone

ADDITIONS	GROWTH*			pH		
	Base	Glycerol	Increase	Base	Glycerol	Decrease
None	18	30	12	7.2	6.0	1.2
Yeast extract, 0.05%	20	40	20	7.2	4.9	2.3
0.1%	24	42	18	7.1	4.9	2.2
1.0%	42	76	34	6.6	4.9	1.7
Accessory factors†	20	38	18	7.2	4.9	2.3
Riboflavin, 5 µg	20	32	12	7.2	5.1	2.1
Fumarate, 0.5%	16	36	20	7.2	5.5	1.7
Fumarate Yeast extract, 0.1%	20	110	90	7.2	5.3	1.9
Fumarate Yeast extract, 0.5%	27	180	153	7.2	5.3	1.9
Fumarate Accessory factors†	15	110	95	7.2	5.3	1.9
Fumarate Riboflavin, 1 µg	20	110	90	7.2	5.4	1.8

\* Turbidity 1 scale unit  $\cong$  6 µg bacterial N per 10 ml.

† Contains: 2.5 µg thiamine; 5 µg each of riboflavin, pyridoxine, and *para*-aminobenzoic acid; 20 µg pantothenic acid; 25 µg nicotinic acid; 1 mµg biotin; and 0.1 µg glutamine per tube.

TABLE 4  
Growth and acid production in synthetic medium

Per tube: 10 ml base medium of Bellamy and Gunsalus (1945) omitting riboflavin and glucose

Incubation: 12 hours, 37 C

RIBOFLAVIN µg/tube	0.5 GLUCOSE		0.5% GLYCEROL		0.5% GLYCEROL 0.5% FUMARATE	
	Growth	pH	Growth	pH	Growth	pH
0	10	7.3	4	7.3	4	7.2
0.1	100	5.6			31	7.0
0.2	100	5.1	20	7.1	46	6.6
0.4	100	5.1	20	7.1	50	6.4
1.0	86	5.1	18	7.3	65	6.1
10.0	90	5.1	20	7.3	56	6.2

*Fermentation Products from Glycerol and Glycerol-Fumarate*

Fermentation balances with *Streptococci faecalis* demonstrated that approximately 95 per cent of the glucose fermented appears as lactic acid (Smith and Sherman, 1942). The fermentation pattern can, however, be altered by alkaline reaction (Gunsalus and Niven, 1942) or with oxidized substrate (Gunsalus and

Campbell, 1944). The fermentation products, with an oxidized substrate such as citric acid, are largely acetic and formic acids and carbon dioxide, with only a trace of lactic acid, thus indicating that while this organism is homofermentative on a balanced substrate, other fermentative potentialities are present. In contrast to the change in products with oxidized substrate, glycerol, a reduced substrate, yields mainly lactic acid in a tryptone yeast-extract base medium (table 5). The two extra hydrogens which arise from glycerol are largely unaccounted for.

A more marked fermentation, accompanied by increased growth, occurs in the presence of glycerol and fumarate. In this case the products are mainly lactic and succinic acids (table 5). In buffered media, especially in the presence of calcium carbonate and an excess of riboflavin, the fermentation can be further altered so that the quantity of fumarate reduced to succinate is greater than the

TABLE 5

*Products of glycerol and of glycerol fumarate fermentation*

Base medium: 1% tryptone, 0.2% yeast extract, 0.2%  $K_2HPO_4$

Incubation: 3 days, 37 C Substrates added aseptically

PRODUCTS	1% GLYCEROL	1% GLYCEROL 1% FUMARATE
	mm/100 ml	mm/100 ml
Lactic acid.....	1.9	3.04
Succinic acid.....	0.46	3.13

Base medium: as above 1%  $CaCO_3$

SUBSTRATES FERMENTED		PRODUCTS FORMED	
	mm/100 ml		mm/100 ml
Glycerol	7.1	Lactate	3.2
		Acetate	2.3
Fumarate	7.5	Succinate	7.5

lactic acid formed (table 5). In this case more oxidized products, acetic acid and carbon dioxide, account for the rest of the glycerol fermented. It is not surprising that under conditions in which fumarate is a good hydrogen acceptor the fermentation is altered in the direction of oxidized products, since it has previously been shown that this organism contains a very active Kreb's dismutation for the formation of acetic and lactic acids and carbon dioxide (Miller, 1942), as well as a system for the conversion of pyruvate to formic and acetic acids (Gunsalus and Campbell, 1944). This would indicate that hydrogen from triose-phosphate, as well as from the glycerol (phosphate), can be transferred to fumarate.

#### DISCUSSION

The anaerobic fermentation of glycerol by streptococci is dependent upon the presence of external hydrogen acceptors, the main pathway of fermentation and energy liberation proceeding by the usual lactic acid pathway. This is contrary

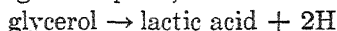
to the results with oxidized substrates in which a series of oxidized products are formed with energy liberation during fermentation. However, the result is similar to that found by Braak (1928) in the colon-aerogenes group. Thus it appears that these organisms are not able to carry out a more reduced type of fermentation than the lactic scheme.

The nature of the hydrogen acceptor of yeast extract that is available to this lactic organism is unknown and might bear investigation. Also, while the mechanism of the fermentation scheme with fumaric acid seems obvious, the nature of the enzymes should be determined; especially since a fumarate reductase (succinoxidase?) system in lactic acid bacteria appears not to have been previously reported.

Taxonomic considerations could call for a review of the relationship of the aerobic and anaerobic glycerol fermentation types of enterococci to *Streptococcus faecium* and *Streptococcus glycerinaceus*, respectively, of Orla-Jensen (1919).

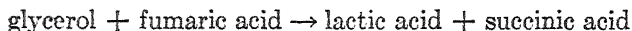
#### SUMMARY

Glycerol fermentation by streptococci has been found to occur only in the presence of external hydrogen acceptors, the main reaction being:



Some strains, as described previously, can use only oxygen as a hydrogen acceptor, the other product being  $\text{H}_2\text{O}_2$ .

Other strains, as reported in this study, can use an unidentified substrate in yeast extract as hydrogenacceptor. This can be replaced by fumaric acid, in which case the main reaction becomes:



This reaction requires a higher riboflavin level than is necessary for glucose fermentation, very probably for hydrogen transport to fumaric acid. With an excess of fumarate, oxidized products are formed.

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# THE "REVERSAL," NEUTRALIZATION, AND SELECTIVITY OF GERMICIDAL CATIONIC DETERGENTS<sup>1</sup>

MORTON KLEIN AND ZELMA G. KARDON

*Department of Bacteriology, School of Medicine, University of Pennsylvania,  
Philadelphia 4, Pennsylvania*

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The reversal of the inhibitory action of antiseptics by substances showing a specific affinity for the inhibiting agent has been reported for many compounds, the most striking effect probably being the reversal of the action of mercurials by H<sub>2</sub>S, glutathione, sodium thioglycolate, etc. Valko and DuBois (1944) have reported that the germicidal action of cationic detergents against both gram-positive and gram-negative bacteria can be reversed by the anionic detergent duponol PC (sodium lauryl sulfate).

In the present work we have studied the ability of anionic detergents to reverse or neutralize the action of cationic detergents. We have found that anionic detergents did not *reverse* the germicidal action of cationic detergents against either gram-positive or gram-negative bacteria. If the cationic detergent inactivated the bacteria, the addition of an anionic detergent did not result in any reactivation. However, when the anionic detergent was added *before* all the bacteria in a given inoculum were inactivated by the cation, the anionic detergent could *neutralize* the action of the cation against *gram-negative* bacteria and prevent any further germicidal action on the surviving bacteria. Against *gram-positive* bacteria the bacteriostatic action of the cationic detergent was not neutralized by an anionic detergent even though the anion was added before the cation. The failure to neutralize the action of a cationic detergent against gram-positive bacteria was found to be related to the high degree of selectivity shown by the compound for gram-positive bacteria.

## EXPERIMENTAL

*Choice of neutralizing agent.* The anionic detergent duponol PC (sodium lauryl sulfate) was used as a neutralizing agent by Valko and DuBois in their studies on the reversibility of the bactericidal action of the cationic detergents. However, in extract broth, pH 7.2, sodium lauryl sulfate was itself inhibitory against *Staphylococcus albus* in dilutions as high as 1:20,000. We therefore examined a group of anionic detergents in an effort to find an agent that was not bacteriostatic against the relatively susceptible gram-positive bacteria but was effective as a neutralizing agent against the cationic detergents. We determined the bacteriostatic action of 12 anionic detergents<sup>2</sup> by seeding various

<sup>1</sup> This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

<sup>2</sup> The anionic detergents tested were aerosol OT, tergitol 7, triton W-30, triton 720, igepon AP, igepon TD, duponol C, sodium octyl, decyl, lauryl, myristyl, and cetyl sulfates. References to the formulae of these compounds and the cationic detergents have previously been given (Klein and Stevens, 1945).

dilutions of the anionic detergents in extract broth, pH 7.2, with 0.1 ml of a 20- to 24-hour broth culture of *S. albus* and determining the degree of inhibition after 24 hours' incubation at 37 C.

The neutralizing action of the compounds against the cationic detergent zephiran was determined by adding various dilutions of the anions to equal volumes of 1:5,000 zephiran broth. The solutions were seeded with 0.1 ml of a 20- to 24-hour broth culture of *Escherichia coli*, and the lowest concentration of anionic detergent inhibiting the action of zephiran was determined. *E. coli* was used as the test strain in the neutralization tests since anionic detergents have little activity against gram-negative bacteria at pH 7.2.

The anionic detergents were found to vary markedly in their ability to neutralize the action of cationic detergents, and there was in general a correlation between the degree of inhibition of *S. albus* and the extent of the neutralization of zephiran against *E. coli*. No compound was found which was completely noninhibitory against *S. albus* and still effective in neutralizing the action of zephiran against *E. coli*. Sodium decyl sulfate was found to be the best neutralizing agent and the only compound which combined a low bacteriostatic activity with a high degree of neutralizing action against *E. coli*. It was only slightly inhibitory against *S. albus* in a 1:5,000 broth dilution, was not inhibitory at a 1:10,000 dilution, and was completely inactive against the gram-negative bacilli in a 1:5,000 broth dilution, pH 7.2. The addition of an equivalent volume of a 1:5,000 dilution of the compound permitted the growth of *E. coli* in a 1:5,000 broth dilution of zephiran. Sodium decyl sulfate was also found to neutralize effectively the action of the cationic detergents ceepryn, phemerol, and emulsol 607 against *E. coli*.

*Failure to obtain reversal of the germicidal action of the cationic detergents.* In our studies on the ability of the anions to reverse the action of cationic detergents, we first repeated the procedure used by Valko and DuBois. Three anionic detergents were used as neutralizing agents: duponol C, a commercial grade of alkyl sulfates, primarily sodium lauryl sulfate; sodium lauryl sulfate; and sodium decyl sulfate. Our test procedure followed that of Valko and DuBois though, in addition to broth subcultures, plate counts were used to determine quantitatively the degree of reversal. To varying dilutions of the cationic detergents in distilled water, 0.5 ml of a 20- to 24-hour broth culture of the test bacteria were added. After 5 minutes at room temperature (several tests were run at 37 C with similar results), 4 loops were subcultured into extract broth and a 1-ml sample was removed and diluted in saline; counts were determined in extract agar. Immediately after the removal of the sample for plating 0.5 ml of the test concentration of the anionic detergent were added to the solution of cationic detergents. Five or 10 minutes (several tests were also done at 30 minutes) after the addition of the anionic detergents, broth subcultures were again made and a 1-ml sample was removed, diluted in saline, and plated out to determine the degree of reversal effected by the anionic detergent. Four test strains were used, *Staphylococcus aureus*, *Escherichia coli*, *Shigella paradysenteriae* (Flexner), and *Salmonella schottmuelleri*. A total of 30 assays were done with



the cationic detergents zephiran and phemerol. Though the dilutions of the cationic detergents and the anionic detergents varied from 1:3,000 to 1:20,000 and the ratio of cation to anion varied from 1:1 to 1:6, in no case was any evidence of reversal obtained in the germicidal range of the cationic detergents. As determined by plate counts, there was never any increase in the number of viable bacteria after the addition of the neutralizing agent. Broth subcultures from the germicidal concentrations of zephiran and phemerol were always negative, and all subcultures remained negative after the addition of the neutralizing agent.

*Neutralization of the action of cationic detergents against gram-negative bacteria.* Though no reversal was obtained with the germicidal concentrations of zephiran or phemerol, it was thought that reversal might be effected with the more dilute concentrations of zephiran in the bacteriostatic range. With sodium decyl sulfate as the neutralizing agent, the following test procedure was used. For each test a duplicate series of broth tubes containing dilutions of zephiran ranging from 1:1,000 to 1:3,000,000 were seeded with 0.1 ml of a 20- to 24-hour broth culture of the test bacteria. After the bacteria were in contact with zephiran for 15 minutes at room temperature, equal volumes of sodium decyl sulfate in a broth dilution of 1:5,000 (1:15,000 and 1:25,000 sodium decyl sulfate were also used against the gram-positive bacteria) were added to one series of tubes, and equal volumes of broth were added to the control zephiran tubes. The cultures were incubated at 37 C for 24 hours, and the titers obtained with the zephiran alone were compared with the titers obtained with the zephiran and sodium decyl sulfate. A total of 17 gram-negative and gram-positive bacteria were studied. As shown in table 1, sodium decyl sulfate differed markedly in its zephiran-neutralizing action when tested against the gram-positive bacteria (and meningococcus) and the gram-negative bacteria. There was only a slight degree of neutralization of zephiran action against 3 of the 10 gram-positive bacteria, but effective neutralization was obtained with all of the gram-negative bacteria.

In order to determine whether growth in broth tubes to which sodium decyl sulfate was added was an actual reversal of zephiran action on the gram-negative bacteria or merely an interruption of the continued action of zephiran (neutralization) the foregoing test procedure was modified as shown in table 2. The data reveal that the growth obtained after the addition of sodium decyl sulfate was not a reversal phenomenon, as indicated by the absence of any significant increase in the bacterial count. Sodium decyl sulfate merely interrupted the continued action of the zephiran; upon further incubation the viable gram-negative bacteria present at 15 minutes in zephiran alone were inhibited, whereas the addition of the sodium decyl sulfate neutralized the zephiran and permitted the surviving bacteria to grow out.

*Failure to obtain neutralization of the action of zephiran against the gram-positive bacteria.* Though viable gram-positive bacteria were present in the high zephiran dilutions at the time of the addition of the sodium decyl sulfate, no significant degree of neutralization was obtained (table 1). Inhibition of growth by the

sodium decyl sulfate was not a factor in the failure to obtain neutralization since a 1:30,000 broth dilution (equivalent to the 1:15,000 sodium decyl sulfate added to an equal volume of zephiran) permitted the growth of an inoculum of 10 to 100 bacteria from a *S. aureus* broth culture. As shown in table 3, neither sodium decyl sulfate nor sodium lauryl sulfate was able to neutralize the *bacterio-*

TABLE 1  
Neutralization of the bacteriostatic action of zephiran broth by sodium decyl sulfate

GRAM-POSITIVE BACTERIA	ZEPHIRAN BROTH*		ZEPHIRAN BROTH PLUS 1:5,000 SODIUM DECYL SULFATE†	
	Inhibited by	Growth in	Inhibited by	Growth in
<i>Staphylococcus aureus</i> 5A.....	1:1,000‡	1:2,000	1:800	1:1,000
<i>Streptococcus pyogenes</i> .....	1:800	1:1,000	1:600	1:800
<i>Bacillus subtilis</i> .....	1:1,000	1:2,000	1:800	1:1,000
<i>Staphylococcus aureus</i> 4A.....	1:1,000	1:2,000	1:1,000	1:2,000
<i>Staphylococcus albus</i> .....	1:800	1:1,000	1:800	1:1,000
<i>Bacillus mycoides</i> .....	1:800	1:1,000	1:800	1:1,000
<i>Sarcina lutea</i> .....	1:2,000	1:3,000	1:2,000	1:3,000
<i>Gaffkya tetragena</i> .....	1:2,000	1:3,000	1:2,000	1:3,000
<i>Diplococcus pneumoniae</i> .....	1:200	1:400	1:200	1:400
<i>Neisseria intracellularis</i> .....	1:400	1:500	1:400	1:500
GRAM-NEGATIVE BACTERIA				
<i>Eberthella typhosa</i> .....	1:40	1:80	1:5	1:10
<i>Shigella paradysenteriae</i> (Flexner)....	1:200	1:400	1:20	1:40
<i>Escherichia coli</i> .....	1:100	1:200	1:5	1:10
<i>Proteus vulgaris</i> .....	1:20	1:40	1:1	1:5
<i>Salmonella schottmuelleri</i> .....	1:100	1:200	1:10	1:20
<i>Salmonella paratyphi</i> .....	1:10	1:20	1:1	1:5
<i>Pseudomonas aeruginosa</i> .....	1:1	1:3		1:1

\* *Neisseria intracellularis*, *Streptococcus pyogenes*, and *Diplococcus pneumoniae* were grown in Difco phenol red glucose broth, pH 7.2. The activity of zephiran in this medium was less than in the extract broth, pH 7.2, used in all other assays.

† A 1:15,000 sodium decyl sulfate broth was also used as a neutralizing agent against all of the gram-positive bacteria and *Neisseria intracellularis*. A 1:25,000 sodium decyl sulfate broth was also used against *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Neisseria intracellularis*. Titers were similar at all concentrations of the neutralizing agents. All readings were taken after 24 hours' incubation at 37 C.

‡ Indicated dilutions  $\times 10^{-2}$ .

static action of zephiran against gram-positive bacteria when added *before* the bacteria. The addition of sodium decyl sulfate to the zephiran before the addition of the *gram-negative* bacteria did result in effective neutralization.

Though the anionic detergent did neutralize the immediate *germicidal* action of zephiran against gram-positive bacteria, the bacteria were not capable of growing. For example, when 1:10,000 or 1:20,000 sodium lauryl sulfate broth was added to equal volumes of 1:10,000 or 1:20,000 zephiran broth and then seeded with *S. aureus*, the rapid *germicidal* action of zephiran was neutralized, but after 24 hours' incubation the viable bacteria failed to grow and plate counts

TABLE 2  
Neutralization of action of zephiran by sodium decyl sulfate  
(*Escherichia coli*)

	ZEPHIRAN BROTH DILUTIONS			
	1:10,000	1:20,000	1:40,000	Broth Control
A. 15 min in zephiran broth. Bacteria per ml	$7 \times 10^2$	$11 \times 10^4$	$3.2 \times 10^6$	$1.5 \times 10^8$
Growth in 24 hr	0	0	0	++++
B. 15 min in zephiran broth. 1:4,000 sodium decyl sulfate broth added. After 15 min bacteria per ml	$17 \times 10^2$	$7.8 \times 10^4$	$5.4 \times 10^6$	
Growth in 24 hr	+++	++++	++++	++++

The titer of zephiran in this assay was lower than it was in previous assays. Similar results were obtained in several assays with *Shigella paradysenteriae* (Fleznar) and *Salmonella paratyphi*.

TABLE 3  
Neutralization of action of zephiran by sodium decyl sulfate  
(*Staphylococcus aureus* strain 4A)

	ZEPHIRAN BROTH DILUTIONS							
	1:25*	1:50	1:100	1:200	1:400	1:600	1:800	Control
Zephiran broth. Growth in 24 hr	0	0	0	0	0	0	0	++++
Zephiran broth plus 1:15,000 sodium decyl sulfate. Growth in 24 hr	0	0	0	0	0	0	0	++++
Zephiran broth plus 1:50,000 sodium decyl sulfate. Growth in 24 hr	0	0	0	0	0	0	0	++++

(*Eberthella typhosa*)

	1:5*	1:10	1:20	1:40	1:80	Control
Zephiran broth. Growth in 24 hr	0	0	0	0	+++	++++
Zephiran broth plus 1:10,000 sodium decyl sulfate. Growth in 24 hr	+++	+++	+++	+++	++++	++++

Equal volumes of zephiran broth and sodium decyl sulfate broth combined before seeding with 1 loop of a 20-hour culture of *S. aureus* or *E. typhosa*. Similar results were obtained with *S. aureus* when 1:25,000 and 1:50,000 sodium lauryl sulfate were used as the neutralizing agent.

\* Indicated dilution  $\times 10^{-2}$ .

made after 6 and 24 hours' incubation showed a gradual reduction in the number of viable bacteria.

*Selectivity of the action of cationic detergents.* On the basis of *germicidal* activity and the inhibition of bacterial respiration (Baker, Harrison, and Miller, 1941) the cationic detergents have been found to be relatively nonselective in their activity against gram-positive and gram-negative bacteria, showing only a slightly greater activity against gram-positive bacteria than against gram-negative bacteria. In the present studies on the bacteriostatic activity of zephiran, however, the compound, as shown in table 1, has, after neutralization, several hundredfold greater activity against the gram-positive bacteria (and *Neisseria intracellularis*) than against the gram-negative bacteria, a selectivity quite similar to inhibitors such as penicillin or gentian violet.

#### DISCUSSION

Our results have shown that anionic detergents are not capable of reversing the action of cationic detergents in a manner analogous to the reversal obtained with  $H_2S$  acting on  $HgCl_2$ . Hotchkiss (1946) has reported that relatively high concentrations of cationic detergents damage the bacterial cell with a subsequent release of the intracellular constituents into the surrounding medium.  $HgCl_2$  did not effect this destruction of the cell. Under such conditions one would not expect any reversal of the action of high concentrations of zephiran, and no reversal was obtained in the present work.

Our results on the selectivity of action of zephiran on gram-positive bacteria indicate that it has essentially the same bacterial spectrum as an agent such as penicillin. The recognition of the selectivity of action of penicillin and the classification of zephiran as a relatively nonselective inhibitor is probably due to the fact that we routinely test our chemotherapeutic agents by means of bacteriostatic tests, whereas germicides are ordinarily assayed by means of the phenol coefficient test. The high concentrations required for the bactericidal action of the compound obscured the selective action in the bacteriostatic range.

The high bacteriostatic activity of zephiran against the gram-positive bacteria and the failure of anionic detergents to neutralize this action indicates a specificity of action not revealed in the studies on the inhibition of bacterial respiration (Baker, Harrison, and Miller, 1941). Gale and Taylor (1946) have found that the assimilation and concentration of glutamic acid is restricted to the gram-positive bacteria and this mechanism is specifically inhibited by penicillin. In view of the similarities in the susceptibility of gram-positive and gram-negative bacteria to penicillin and zephiran, it is possible that the relatively selective gram-positive bacteriostatic activity of zephiran may be related to a similar mechanism.

#### SUMMARY

The activity of the cationic detergents zephiran and phemerol against gram-positive and gram-negative bacteria could not be reversed by the anionic detergents sodium decyl sulfate or sodium lauryl sulfate.

Anionic detergents neutralize the bacteriostatic action of zephiran against gram-negative bacteria but do not neutralize the bacteriostatic action of zephiran against gram-positive bacteria.

The cationic detergent zephiran in its bacteriostatic range has a high degree selectivity and possesses several hundredfold greater activity against gram-positive bacteria than against gram-negative bacteria.

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# TUBERCULOSTATIC AND TUBERCULOCIDAL PROPERTIES OF STREPTOMYCIN<sup>1,2</sup>

DOROTHY G. SMITH AND SELMAN A. WAKSMAN

*New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick,  
New Jersey*

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Antibiotics are frequently looked upon as primarily bacteriostatic agents, in spite of the fact that their bactericidal properties have been definitely established. The reasons for such assumptions are dependent largely upon the following characteristic properties of antibiotic substances: 1. Compared to chemical antiseptics, antibiotics exert a much slower bactericidal effect upon sensitive organisms. 2. The bactericidal action of an antibiotic depends upon a number of factors, as, for example, the age of the bacterial culture; penicillin is largely bacteriostatic against old cultures but it is bactericidal as well for young, rapidly growing cultures of bacteria. 3. Frequently a much larger concentration of the antibiotic is required to kill the bacterial cells than is necessary to inhibit their growth. 4. When a few viable cells are left in the culture to which the antibiotic has been added, especially when those cells are more resistant to the action of a given concentration of the antibiotic, they begin to develop rapidly, giving rise to a more resistant culture; the impression may thus be produced that the antibiotic has only a limited bactericidal effect.

In a comparative study of the bactericidal action of a number of antibiotics (Waksman and Reilly, 1944), the conclusion was reached that those agents which are characterized by a high bacteriostatic action against a certain organism are also strongly bactericidal. This action depends upon both the nature of the organism and that of the antibiotic.

The use of streptomycin in the chemotherapy of tuberculosis has recently focused particular attention on the problem of the bacteriostatic vs. bactericidal action of this antibiotic. The fact that tubercular infection in experimental animals is not rapidly eliminated by treatment with streptomycin, an antibiotic which possesses marked antituberculosis properties (Schatz and Waksman, 1944), gave the impression that this antibiotic acts largely as a bacteriostatic rather than as a bactericidal agent (Hinshaw and Feldman, 1945). This conclusion was based primarily on the fact that streptomycin does not bring about complete sterilization of the animal body. Upon cessation of streptomycin therapy, the few remaining cells which in the meantime may have developed resistance may begin to multiply again. A state of reinfection may thus be brought about, giving the impression that the antibiotic did not exert any bactericidal action at all.

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It has been amply demonstrated (Waksman, 1947), however, that various antibiotics exert a marked bactericidal effect upon the gram-positive and gram-negative bacteria. Although the early demonstration (Schatz and Waksman, 1944) of the bactericidal action of streptomycin may be considered as inconclusive (Middlebrook and Yegian, 1946), lack of suitable methods for the enumeration of tubercle bacteria prevented at that time the presentation of results in more accurate form, although there was no doubt that the antituberculosis effect of the antibiotic was also markedly bactericidal. By the use of a rather crude technique it was demonstrated that when 200 to 300  $\mu$ g per ml of streptomycin were allowed to act upon clumps of the human strain of the tubercle organism (H37) for several days at 37 C, there was a striking reduction in the number of viable cells.

Streptothricin, an antibiotic closely related to streptomycin, was also found (Woodruff and Foster, 1944) to exert a tuberculocidal action, three units of streptothricin per milliliter of culture being required to kill all the cells of the nonpathogenic strain of *Mycobacterium tuberculosis* no. 607 in 14 days; a bacteriostatic effect was brought about by only 0.3 to 1.0 unit.

The bactericidal action of streptomycin upon the tubercle organism in experimental infections as well as in clinical tuberculosis has also been established. This hardly justifies the previous statement that streptomycin is largely tuberculostatic and not tuberculocidal. This was well expressed recently by Dr. Feldman (1947): "Evidence obtained from clinical sources indicates quite definitely a marked diminution in the number of tubercle bacilli that can be demonstrated by cultural means from such materials as bronchial secretions, gastric washings and urine after the patient has been under treatment with streptomycin for some time."

Recently, suitable media have been developed (Dubos and Davis, 1946) for obtaining diffuse growth of *M. tuberculosis* throughout the culture. This permitted the use of accurate turbidimetric methods (Smith, 1947) for the quantitative estimation of the growth of the organism and for the evaluation of the effect of antibiotics upon the course of its multiplication. When agar is added to such media, the exact number of living cells of *M. tuberculosis* in a given suspension can be determined, thus making it possible to measure accurately both the tuberculostatic and the tuberculocidal action of an antibiotic, and to interpret more closely the relative significance and the interrelations of these two phenomena in the survival of this organism in an artificial culture or in the body of the host.

#### EXPERIMENTAL

*Organisms and methods.* The following investigations were undertaken for the purpose of clarifying the bacteriostatic vs. the bactericidal effects of streptomycin upon certain acid-fast bacteria. For this purpose, various saprophytic and pathogenic organisms were used. Most of the cultures, with the exception of the two human pathogens, were obtained from the American Type Culture Collection.



These *Mycobacterium* cultures were *M. phlei*, the so-called timothy hay bacillus; *M. avium*; *M. tuberculosis* var. *hominis* no. 607, nonpathogenic strain; *M. tuberculosis* var. *hominis* H37Rv, pathogenic strain; *M. tuberculosis* var. *hominis* H37RvR, streptomycin-resistant pathogenic strain obtained from Dr. Youmans.

The method of growing the organisms for bacteriostatic tests has been described elsewhere (Smith, 1947). For bactericidal studies, the cultures were plated out on suitable agar media, incubated for varying periods of time at 37 C, and all the colonies counted.

*Bacteriostatic and bactericidal action of streptomycin.* The results of several experiments are reported in order to demonstrate the relationship between the antituberculosis effect of streptomycin, its concentration, and the length of the incubation period. In the first two experiments, the nonpathogenic strain of

TABLE 1  
*Bacteriostatic effect of streptomycin on Mycobacterium tuberculosis no. 607*

INCUBATION	STREPTOMYCIN, MICROGRAMS PER MILLILITER						
	0.0	0.1	0.2	0.3	0.5	1.0	2.0
	Turbidimetric readings (in logs)						
<i>hours</i>							
6	0	0	0	0	0	0	0
12	3	3	0	0	0	0	0
24	18	13	3	0	0	0	0
48	45	31	7	0	0	0	0
72	53	48	10	0	3	0	0
240	155	158	112	0	4	0	0

*M. tuberculosis* was used. When a short incubation period of 12 hours is used, 0.2  $\mu$ g per ml is sufficient to inhibit the growth of the organism. When incubation of the cultures is continued for a longer period, however, 0.3  $\mu$ g per ml becomes the inhibiting concentration. In one set of cultures a limited amount of growth was obtained upon prolonged incubation, even in the presence of 0.5  $\mu$ g per ml of the antibiotic (table 1).

The bactericidal effect of streptomycin upon the nonpathogenic strain is shown in table 2. Here as well, the incubation period was found to be of great importance. Although a certain bactericidal action was obtained even with 0.3  $\mu$ g per ml of the antibiotic, especially after a longer incubation period, it took more than 2  $\mu$ g per ml to kill all the cells in 6 hours; a similar effect was exerted by 1  $\mu$ g per ml when incubation was continued 24 hours. With lower concentrations of streptomycin, it took more than 24 hours to destroy all the cells, as detected by the plate method. The smallest concentration of streptomycin, namely, 0.2  $\mu$ g per ml, exerted chiefly a bacteriostatic effect.

The pathogenic strain of *M. tuberculosis* H37Rv grows more slowly. More streptomycin was required to inhibit its growth than was the case with the non-

pathogenic strain: it took 2  $\mu$ g per ml of streptomycin to bring about complete inhibition, and 1  $\mu$ g per ml, for partial inhibition (table 3).

In view of the fact that it is generally recognized that the size of the inoculum has a decided influence upon the antibiotic effect of a given agent, a study was made of the significance of the inoculum size upon the tuberculostatic vs. tuber-

TABLE 2

*The bactericidal activity of streptomycin on Mycobacterium tuberculosis no. 607*

INCUBATION	STREPTOMYCIN, MICROGRAMS PER MILLILITER					
	0.0	0.2	0.3	0.5	1.0	2.0
	Numbers of viable cells per milliliter*					
hours						
6	283,000	229,000	83,800	26,700	1,120	40
12	1,800,000	427,000		3,000	490	0
24	18,900,000	1,660,000	200	220	0	0
48	12,650,000	1,770,000		700	0	0
72	16,500,000	3,944,000	0	2,500	0	0
240	9,400,000	6,330,000	0†	7,500	0	0

\* Number at start, 216,000.

† In a duplicate series, 20,900,000 cells per ml were found after 240 hours' incubation in the presence of 0.3  $\mu$ g per ml of streptomycin.

TABLE 3

*The bacteriostatic action of streptomycin on Mycobacterium tuberculosis var. hominis strain H37Rv*

	STREPTOMYCIN, MICROGRAMS PER MILLILITER				
	0.0	0.6	1.0	2.0	4.0
	Turbidimetric readings (in logs)				
days					
1	7	1	0	2	2
4	42	16	10	6	4
7	83	37	12	5	4
11	130	68	12	0	0
14	186	92	13	0	0

culocidal properties of streptomycin. For this purpose, *M. avium* was used. The results (table 4) show that there is a definite direct correlation between the size of inoculum and the amount of streptomycin required to inhibit the growth of the organism. More of the antibiotic was required to inhibit *M. avium* when 0.1 mg per ml of inoculum was used than with only 0.001 mg per ml or less of the cell material. The same was true for the bactericidal action of streptomycin: it took four times as much streptomycin to inhibit the growth of, or to kill, all the cells capable of developing on the plate when 0.1 mg per ml of the inoculum was used than when 0.00001 mg per ml was used.

Development of resistant cells in cultures containing varying amounts of streptomycin is shown by the results of a typical experiment (table 5). As

TABLE 4

*Influence of inoculum on the bacteriostatic and bactericidal action of streptomycin on Mycobacterium avium*

INCUBATION	CONCENTRATION OF CELL MATERIAL IN MG/ML			
	0.1	0.01	0.001	0.00001
$\mu\text{g/ml}$ of streptomycin required for bacteriostasis				
<i>days</i>				
1	0.4	0.4	0.05	0.0
3	0.6	0.4	0.4	0.2
7	0.8	0.4	0.4	0.2
$\mu\text{g/ml}$ of streptomycin required for bactericidal action*				
1	>1.0	>1.0	1.0	0.6
3	0.8	0.4	0.4	0.4
7	0.8	0.4	0.4	0.2

\* Amounts required to give complete destruction of all cells.

TABLE 5

*Influence of age of culture of Mycobacterium tuberculosis no. 607 upon the development of cells resistant to different concentrations of streptomycin*

AGE OF CULTURE	NUMBER OF CELLS (THOUSANDS/ML) IN MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF STREPTOMYCIN ( $\mu\text{G/ML}$ )			
	0.0	0.3	0.5	0.7
<i>hours</i>				
0	30	10	0	0
2	32	10	0.2	0.01
4	33	0	0	0
6	35	17	0	0
22	3,600	1,550	6.6	0
30	13,560	5,050	3.6	0.4
42	21,800	2,500	70.0	0.6
48	40,450	11,400	10.0	0.3
66	75,100	2,600	800.0	1.4
89	130,000	19,500	2.1	3.7
96	143,000	7,200	2.2	2.5
114	425,000	12,680	5.8	5.4
168	650,000	25,000	38.3	5.8
235	503,500	1,300	1.6	7.9
336	1,130,000	28,200	15.0	6.5
504	561,000	63,500	570.0	40.7

cultures developed, there was actually a decrease rather than an increase in the proportion of resistant cells. The percentage of cells resistant to 0.3  $\mu\text{g}$  per ml of streptomycin decreased from 30 per cent at the start to 15 per cent or less

after 48 hours' incubation. In a growing culture the percentage of resistant cells to larger amounts of streptomycin was even less, the sensitive cells not being eliminated at the expense of the resistant cells.

*The combined effect of two antibiotics upon M. tuberculosis.* Simultaneously with the extensive application of streptomycin in the treatment of clinical tuberculosis, certain important problems have arisen. None of these is of greater significance than the development of resistance of bacteria to this antibiotic. Fortunately, an organism that has become resistant to one antibiotic does not necessarily become resistant to another. This has been well illustrated, for example, in the case of bacteria sensitive to both penicillin and streptomycin. The utilization of the synergistic action of two antibiotics in order to eliminate completely all the cells of a given bacterium has, therefore, been suggested. That this is possible for certain antibiotics active against gram-negative bacteria has already been demonstrated (Waksman and Reynolds, 1947). Its application

TABLE 6

*Relative bacteriostatic effect of streptomycin and streptothricin on streptomycin-sensitive and streptomycin-resistant strains of Mycobacterium tuberculosis var. hominis*

STRAIN	SENSITIVITY TO STREPTOMYCIN	INCUBATION  days	$\mu$ g/ml of antibiotic required for growth inhibition*	
			Streptomycin	Streptothricin
Avirulent 607	Sensitive	7	0.2	0.8
H37Rv	Sensitive	14	2.0	8.0
H37Rv	Resistant	14	>10,000	600.0

\* The inoculum consisted of 0.01 mg per ml of cell material.

to the treatment of experimental tuberculosis has also been indicated (Smith and McClosky, 1945).

The proper combination of two antibiotics or of one antibiotic and one chemical antiseptic was found to result in the killing of more of the bacterial cells than did either of the agents alone. This synergistic effect holds true only for cases where the cells of an organism made resistant to one agent still remain sensitive to the other.

To determine whether this holds true for the action of streptomycin upon *M. tuberculosis*, this antibiotic was first combined with streptothricin. Three cultures were used in the experiment: the nonpathogenic no. 607, the pathogenic no. H37Rv, and the streptomycin-resistant strain of the last organism (table 6).

Both the avirulent and the virulent but streptomycin-sensitive strains of *M. tuberculosis* require four times as much streptothricin as streptomycin for complete inhibition of growth. The streptomycin-resistant strain of the organism did not remain sensitive to streptothricin, although much less streptothricin than streptomycin was required to inhibit growth of this strain. The fact that 600  $\mu$ g per ml of streptothricin were necessary to inhibit the growth of the streptomycin-resistant strain would tend to remove streptothricin automatically from the field of practical utilization as a potential supplement to streptomycin.

When streptomycin was combined with streptothricin, the effect upon *M. tuberculosis* was additive rather than synergistic, i.e., the use of 1  $\mu$ g each of the two antibiotics per 1 ml of culture medium was equivalent to the action of 1.25  $\mu$ g of streptomycin, namely to a simple arithmetical addition of the potency of the two antibiotics. In the case of a synergistic effect the second antibiotic would be expected to affect the few cells that remained resistant to the action of the first antibiotic. If that were the case, it would have taken comparatively little of the supplementary antibiotic to eliminate the cells of the pathogen from a culture and presumably also from the body of the host.

*Effect of streptomycin upon morphology and acid-fastness.* The morphology and staining properties of an organism are always valuable criteria for the evaluation of the action of any drug on a particular organism. Since these characteristics are based upon the chemical constitution of the cell, any change brought about by a drug in the morphology of the organism or its reaction to stains is indicative of a change in the structure or metabolism of the cell.

The tubercle bacilli have been described (Topley and Wilson, 1936) as rod-shaped organisms, 1 to 4  $\mu$  long and 0.2 to 0.8  $\mu$  broad, straight or slightly curved, with parallel or irregular sides and rounded ends; arranged singly or in small clumps; nonmotile, nonsporing, and noncapsulated. They stain with difficulty, but, when once stained, they are acid-fast.

Of the variety of staining procedures tried on tubercle bacilli (Corper, 1926a), the Ziehl-Neelsen stain, using hot carbol fuchsin, sulfuric acid, and a methylene blue counterstain, has been found to be best. With this stain, the tubercle bacilli appear as red cells, while the non-acid-fast organisms are blue. Depending upon the age of the culture, differences in the densities of the stain taken up by the cells have been observed (Corper, 1926b). Young cells, which are long, straight, or curved filaments, stain uniformly; shorter bacillary adult forms show metachromatic granules, and senescent forms are uniformly stained but are coccoid or very short bacilli.

For the study of the effect of streptomycin on the morphology and acid-fastness of tubercle bacilli, a modification of the Ziehl-Neelsen stain developed by Alexander-Jackson (1944) was used. This modification differs from the original stain in that the counterstaining with methylene blue is followed by a process of decolorization and restaining with a mixture of acid green and acid yellow. In the resulting preparation acid-fast organisms appear red, partly acid-fast are mulberry to blue depending upon the degree of acid-fastness, and other organisms or debris are light green. This gives greater scope to the determination of the effect of an agent on the acid-fastness of a culture.

The principal effects of streptomycin on the morphology of such cultures as *M. avium* and *M. tuberculosis* grown in Tween medium were loss of acid-fastness increase in granulation, and at times, especially in highly bacteriostatic concentrations, in shortening of the bacilli. A progressive loss in acid-fastness occurred with increasingly tuberculostatic quantities of the drug. This reaction was evident in the presence of concentrations of the antibiotic less than the bacteriostatic levels. Beading or granulation followed the same general trend as the loss in acid-fastness; however, this phenomenon did not seem to appear to any

significant degree except with concentrations of streptomycin which produced detectable bacteriostasis. Shortening of the bacilli or coccobacilli was observed only in those concentrations of the drug which were highly inhibitory to growth or were ultimately bactericidal. Chains of more than two or three bacilli and curving of the rods were found only occasionally. No thickening of the cells was observed. These morphological changes followed the same pattern with regard to the effect of the number of cells as did the other conditions of bacteriostatic and bactericidal action. For example, the smaller the number of cells, the smaller was the amount of streptomycin necessary to bring about malformation of the organisms. In concentrations of streptomycin which were only moderately bacteriostatic, all gradations of acid-fast staining were seen. Completely acid-fast rods occurred among the clumps of almost entirely non-acid-fast ones. The question arises as to the nature of those organisms which did not undergo any transformation. It is possible that they are representative of the cells which are resistant or develop resistance to the antibiotic. In general, no completely acid-fast organisms were observed in strongly bacteriostatic or bactericidal concentrations of the drug.

Morphological changes have been reported as being produced by such substances as toluene, chloroform, thymol, ether (Laporte, 1942), and aryl-sulfamides (Courmont, Morel, Perier, 1938) and by poor environmental conditions (Severens and Tauner, 1945; Vera and Rettger, 1940). The organisms were reported to have an increasing tendency to grow in clumps and were altered in form. Granulation inside the cell was greater; the granules were liberated by the dissolution of the ectoplasm of Legroux. Loss of acid-fastness and progressive fragmentation of the free granules were also observed. All of these findings agree with the observation made on cultures under the influence of streptomycin.

When *M. tuberculosis* was grown in different media containing peptone, casein, beef extract, serum albumin, amino acids, inorganic compounds, or glycerol, normal acid-fast cells were found only in those media which contained the more complex forms of nitrogen. When the source of nitrogen was asparagine or ammonium citrate, the cells showed abnormalities, such as loss in acid-fastness and shortening and curving of the bacilli. The effects of streptomycin on the morphology of the tubercle bacilli in the various media were the same as those described for the cells in the Tween medium, namely, progressive loss in acid-fastness with increasing amounts of the drug, increase in beading, and shortening of the rods. In poor media the loss in acid-fastness was so great that the cells stained green, with blue- or mulberry-colored granules. Even in those cultures which appeared to contain no acid-fast organisms, the cells were still viable and regained their normal staining reaction on subculture to glycerol nutrient agar. In short, the damage to the cells produced by streptomycin could not be neutralized by the presence of any of the nutrients used.

#### SUMMARY

Streptomycin has not only a bacteriostatic but also a marked bactericidal action upon different strains of *Mycobacterium tuberculosis*.

The size of the inoculum and the time of incubation are of great importance in determining the bacteriostatic and bactericidal activity of the antibiotic.

In a growing culture of tubercle bacilli, there was a decrease rather than an increase in the proportion of streptomycin-resistant cells with an increase in age of the culture.

When streptomycin and streptothricin were combined, their effect upon tubercle bacilli was additive rather than synergistic.

The principal effects of streptomycin on the morphology of tubercle bacilli were loss of acid-fastness, increase in granulation, and, in highly bacteriostatic concentrations, shortening of the rods.

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## NOTES

### REVERTING HISTOPLASMA CAPSULATUM TO THE YEAST PHASE

CHARLOTTE C. CAMPBELL

*Department of Bacteriology, Army Medical Department Research and Graduate School,  
Washington 12, D. C.*

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The life cycle of the pathogenic fungus *Histoplasma capsulatum* can be completed outside the animal body through cultivation on blood agar incubated in a moist atmosphere at 37 C (Conant: J. Bact., **41**, 563). Many workers have found that some strains which had been cultured in the mycelial phase over long periods of time failed to revert to the yeast phase when cultivated under the conditions specified. When it was found in this laboratory that Francis' glucose cystine blood agar luxuriantly supported growth of the yeast phase of *Sporotrichum schenckii* (Campbell: J. Bact., **50**, 233), other systemic fungi known to exist in the yeast phase in human tissue were also observed on this medium. Many strains of *Histoplasma capsulatum* which had been cultured in the mycelial phase for several years were reverted to the yeast phase without appreciable difficulty.

The AMS modification of the medium originally devised by Francis has proved more satisfactory than any other medium used, and so its preparation is described.

Veal infusion (double strength).....	1,000 ml
Rabbit or horse blood.....	80 ml
Peptone.....	10 g
Glucose.....	10 g
Sodium chloride.....	5 g
Cystine or cystine hydrochloride.....	1 g
Agar.....	20 g

The agar, sodium chloride, and peptone are added to the veal infusion and heated until the agar is dissolved. The cystine is dissolved in the sodium hydroxide solution required to adjust the medium to pH 7.6 to 7.8 and then added to the base mixture. After sterilization at 121 C for 20 minutes, the agar base is cooled to 50 C and the blood and glucose solution added aseptically. The completed medium is maintained at 60 C for 3 hours, thoroughly mixed at intervals, and is then dispensed in tubes or plates.

*Histoplasma capsulatum* can be easily cultured, harvested, and utilized in bacteriologic techniques when maintained in the yeast phase of growth. Several serial transfers may be necessary for the reversion of old stock strains that have been maintained in the mycelial form. Such strains should be transferred serially to new slants of the medium at 2- to 3-day intervals even though no

reversion is apparent. After 3 to 7 transfers, the appearance of small yeast colonies will be noted among the predominating mycelial types. By proper colony selection, the pure yeast phase can be secured. Once reverted, the organism can be maintained in this phase indefinitely by incubation at 37 C. During the reversion process, old slants should not be discarded, but should be continuously incubated at 37 C and observed at daily intervals for papillate colonies in the yeast phase.

## THE VIABILITY OF YEAST CULTURES PRESERVED UNDER MINERAL OIL

B. S. HENRY

*Department of Microbiology, School of Medicine, University of Washington, Seattle,  
Washington*

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At the time that the work of Morton and Pulaski (J. Bact., **35**, 163) on the preservation of bacterial and yeast cultures appeared, we were concerned with the maintenance of a large number of stock cultures of yeasts used in the preparation of experimental wines (Henry: Univ. Wash. Bull., 1937).

Briefly, the method consists of inoculating agar slants in the usual manner, incubating until good growth is obtained, and adding sufficient sterile mineral oil to cover the tip of the slant with at least one-fourth inch when the tube is upright. The cotton stopper is replaced and the culture kept at room temperature. The culture medium used for our yeast cultures was a wort agar of the following composition: malt extract, 100 g; water to make 1,000 ml; and agar, 17 g. The pH falls to about 4.5 without adjustment.

In October, 1939, 17 cultures of yeasts used in the preparation of European and American wines and a large number of unidentified strains isolated from various fermenting fruits were preserved by the method of Morton and Pulaski. In October, 1940, these cultures were transplanted to a similar medium and again covered with sterile mineral oil. This second set of cultures was stored at room temperature and left undisturbed until December, 1946, when the 17 cultures of wine yeasts and a random culture from each of the 8 groups isolated from fermenting fruits were examined and subcultured for viability.

All cultures appeared in good condition and many showed a white to tan, filamentous growth extending out from the slant into the mineral oil. Upon microscopic examination this growth was found to be made up of pseudomycelium and some single cells. Upon subculture on wort medium, growth appeared within 24 hours in most cases, and all subcultures showed abundant growth in 48 hours. Microscopically the cultures showed little or no mycelial growth and agreed with the descriptions made 7 years previously.

# REPORTED SALMONELLAS FROM THE PACIFIC 1941-1946

PHILIP R. CARLQUIST

*Department of Bacteriology, Army Medical Department Research and Graduate School, Army Medical Center, Washington 12, D. C.*

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During the war years several types of *Salmonella* were reported in Japanese and English journals from the Pacific area. Several of these have been indexed in various abstract journals.

*Salmonella chiba* XXVIII: — —, *S. nipponbasi* (XXVIII) XXXIV: z<sub>23</sub> —, and *S. yodobasi* ??? were reported by Ohasi (Japan. J. Exptl. Med., **24**, 1431). These strains have been thoroughly studied and none of them conform with the accepted biochemical pattern established for the genus *Salmonella*. *S. chiba* is nonmotile and utilizes salicin with the production of acid and gas. *S. nipponbasi* produces indole and is methyl-red-negative. Its H antigen is not agglutinated by single factor z<sub>23</sub> serum. *S. yodobasi* conforms closely to the biochemical pattern of *Escherichia coli* and fails to grow on all selective media designed to inhibit the growth of *E. coli*.

*S. kanda* III,X,XXVI: e,h 1,w... was reported by Ohasi (Japan J. Exptl. Med., **27**, 1110) and *S. taihoku* III,X,XXVI: l,w...1,5... was reported by Kurimoto and Tukitari (Japan Med., **3372**, 422). The antigenic pattern of *S. kanda* was found to be as reported and identical with the pattern of *S. mel-eagridis* reported by Bruner and Edwards (Am. J. Hyg., **34**, 82). The true antigenic pattern of *S. taihoku* was also found to be identical with that of *S. meleagridis*.

A new type, *S. singapore* VI,VII: k e,n,x... was isolated from cases reported as acute enteric fever by Hayakawa in 1944. This type has not been reported previously.

*S. iwo-jima*, VI,VIII... i 1,5..., *S. oahu*, IV,V,XII... l,v... 1,2,3..., and *S. saipan*, III,X,XXVI z<sub>6</sub> 1,6... were reported by Lindberg and Bayliss (J. Infectious Diseases, **79**: 91). Of these three types, the strain reported as *S. iwo-jima* has been available for study in this laboratory. Upon antigenic analysis, confirmed by Edwards, this strain was determined to have the pattern VIII,XX i z<sub>6</sub>..., which is the pattern of *S. kentucky* reported by Edwards (J. Hyg., **38**, 306). *S. saipan* was reported as having factor z<sub>6</sub>... as a phase 1 antigen for the first time, and it is unfortunate that a strain is not available for further study.

It is suggested that new types within the genus *Salmonella* should be verified by independent laboratories before publication in order to avoid further confusion in the classification of the *Salmonella*.

# ANAEROBIC FERMENTATION OF MANNITOL BY STAPHYLOCOCCI

JAMES B. EVANS

*Laboratory of Bacteriology and School of Nutrition, Cornell University, Ithaca,  
New York*

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Mannitol fermentation has long been considered an important test in studying staphylococci, and yet reports on its correlation with other tests vary widely. Hallman (Proc. Soc. Exptl. Biol. Med., **36**, 789) found a 91 per cent correlation with the coagulase test on 487 strains. On the other hand, Plastringe *et al.* (Storrs Agr. Expt. Sta., Bull. 231) found that 74 per cent of their 211 coagulase-negative strains fermented mannitol. Colwell (J. Bact., **37**, 245) reported that of 28 mannitol-fermenting strains only 2 would ferment anaerobically. Unfortunately, her tests were not correlated with the coagulase test.

Study of a small collection of mannitol-fermenting staphylococci has revealed that the anaerobic fermentation of mannitol correlated 100 per cent with the coagulase test (see table).

	NO. OF CULTURES	FINAL pH IN MANNITOL BROTH	
		Anaerobic	Aerobic
Coagulase (+)	11	(10) 5.0-5.6 (1) 6.2	4.9-5.2
Coagulase (-)	21	7.0-7.2	5.0-5.9

The group of coagulase-positive cultures included 4 from clinical infections, 2 old stock cultures, and 5 from frozen foods. The coagulase-negative strains were all from frozen foods.

It would seem from this that the incorporation of mannitol in selective aerobic plating media is useful, but not perfect. In addition, it has been noted that when 7.5 per cent NaCl is included in the plating medium (Chapman: J. Bact., **50**, 201) the acid production by coagulase-positive strains is reduced, but not, in a majority of cases, that of the coagulase-negative strains. This is so pronounced that, if bromcresol purple is substituted for the phenol red indicator, the coagulase-positive strains produce very little or no yellow zone. Of the 21 coagulase-negative strains, 14 produced a pronounced yellow zone on this medium.

It is recognized that only a small collection of organisms from a limited number of sources has been used, and it is scarcely to be expected that the correlation between the coagulase test and anaerobic fermentation of mannitol will remain perfect, but the importance of anaerobiosis should be noted. This may serve to re-evaluate the importance of mannitol fermentation in studying staphylococci.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## NEW JERSEY BRANCH

NEW BRUNSWICK, NEW JERSEY, MARCH 13, 1947

PRELIMINARY OBSERVATIONS ON THE GROWTH REQUIREMENTS OF *BACILLUS POPILLIAE* DUTKY AND *BACILLUS LENTIMORBUS* DUTKY. *S. R. Dutky*, U. S. Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Moorestown, New Jersey.

*Bacillus popilliae* Dutky, causal agent of type A milky disease of Japanese beetle larvae, has been maintained under continuous cultivation on artificial media for a year. Some media gave consistently high yields of vegetative forms, but none was adequate for sporulation. Cultures carried through numerous transfers produced typical disease symptoms and abundant spores when injected into Japanese beetle larvae. Factors

affecting culture yields were carbohydrate content, pH, buffer capacity, reducing capacity, and thiamine content of the medium. Glucose and fructose served as sources of energy, whereas peptones, sucrose, lactose, galactose, or glucosamine did not. About 250 million cells were produced per mg of glucose fermented. *B. popilliae* made best growth at pH 7.5 in highly buffered strongly reducing media. Thiamine was essential; about 0.003  $\mu$ g per 10 ml of medium supplied enough for good growth. Preliminary tests indicated that *B. popilliae* may be useful for the assay of minute amounts of thiamine.

*Bacillus lentimorbus* Dutky, causal agent of type B milky disease, was cultured similarly.

## SOUTHERN CALIFORNIA BRANCH

LOS ANGELES, CALIFORNIA, MARCH 18, 1947

ANAEROBIC HYDROGEN TRANSFERS AFFECTING FATTY ACIDS. *William D. Rosenfeld*, Scripps Institution of Oceanography, University of California, La Jolla, California.

The bacterial dehydrogenation of saturated and unsaturated monocarboxylic acids has been followed by means of the Thunberg technique. Lipoclastic anaerobes isolated from petroliferous materials rapidly dehydrogenated formic acid, whereas the oxidation of other volatile acids was sporadic. Saturated acids ranging from valeric through stearic were not attacked, nor were the unsaturated acids: undecylenic, oleic, linoleic, and linolenic. Obligately anaerobic sulfate-reducing bacteria obtained from marine muds dehydrogenated a wider range of fatty acids, although their activities were restricted to compounds containing even numbers of carbon atoms. Unsaturated  $C_{18}$  acids were also dehydrogenated.

The reduction, or hydrogenation, of fatty acids was demonstrated by means of a re-

verse Thunberg method in which leucomethylene blue was oxidized in the presence of compounds activated as hydrogen acceptors. Complete recoloration of the dye did not occur, a circumstance probably related to the comparatively high Eh level required for such oxidation. Both saturated and unsaturated acids were susceptible to reduction by lipoclasts. This group of acids extended from  $C_2$  through  $C_{18}$ . Reduction products were not identified.

Oleic, linoleic, and linolenic acids were activated as hydrogen acceptors in the dehydrogenation of formic acid. The reactions were measured in Warburg respirometers. Molar concentrations of the unsaturated acids added were inversely proportional to their degrees of unsaturation, while the concentration of formic acid remained constant. The amount of carbon dioxide produced in all cases approached 20 per cent of that which would result from the complete oxidation of formic acid.

## PHENOL PRODUCTION BY MARINE BACTERIA.

David M. Updegraff, Scripps Institution of Oceanography, La Jolla, California.

Over 90 per cent of 67 samples of marine sediments examined have yielded enrichment cultures that actively decompose *l*(-)-tyrosine, either as the free amino acid or combined in Difco neopeptone or casein, with the production of either phenol or *p*-cresol, or occasionally mixtures of the two.

The reaction proceeds well under anaerobic conditions and also when air is present over the surface of the medium, but not when air is bubbled through the medium. In from 16 to 24 hours' incubation at 27 C, a medium containing 0.025 per cent of *l*(-)-tyrosine and 0.1 per cent peptone in sea water at pH 7.1 to 8.2 yielded over 50 per cent of the theoretical amount of phenol, assuming that one mole of tyrosine yields one mole of phenol. In a medium containing 0.025 per cent of *l*(-)-tyrosine as the sole organic carbon source, an equally good yield of phenol was obtained in 48 hours. Phenol is formed more often from free tyrosine when some access to air is permitted. *Para*-cresol is formed more often from casein or neopeptone under anaerobic conditions.

Several pure cultures capable of producing phenol from tyrosine have been isolated. They are all motile, nonsporulating, gram-negative rods, 1 to 1.5 by 1.5 to 5 microns. They form round entire colonies with no pigment on agar. Some cultures will grow on tyrosine as the sole organic carbon source. None produce phenol from *p*-hydroxybenzoic acid, thus differing from *Escherichia coli-phenologenes*.

THE ANTAGONISTIC EFFECT OF *BACILLUS CEREUS*. R. J. Goodlow, C. W. Johnson, and M. V. Shafer, Department of Bacteriology, University of Southern California, Los Angeles, California.

A strain of *Bacillus cereus*, isolated from milk, exhibits marked antagonistic activity against both gram-positive and gram-negative bacteria. Pour plates of proteose peptone agar were prepared by adding test organisms in a dilution of 300,000,000 bacteria per ml. After the medium had solidified, *B. cereus* was streaked across the surface of the plates. Cultures were incubated at 30 C and observed at intervals of 6,

12, 24, 48, and 72 hours. After a period of 6 hours' incubation, marked zones of inhibition of growth occurred in cultures of *Corynebacterium pseudodiphtheriae*, *Salmonella anatum*, *Salmonella typhi-murium*, *Salmonella paratyphi*, *Shigella ambigua*, *Serratia marcescens*, *Neisseria catarrhalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus mesentericus*, and *Bacillus mycoides*. At the end of 24 hours of incubation all of these organisms, with the exception of *S. ambigua* and *S. paratyphi*, exhibited zones of growth stimulation peripheral to the zone of growth inhibition. This was especially marked in *B. mycoides*, which developed, after 72 hours, four alternate zones of inhibition and stimulation. After 48 hours of incubation, plates of *Shigella flexneri* I and *Mycobacterium* sp. showed excellent zones of growth inhibition adjacent to the *B. cereus* colonies.

No inhibition of growth occurred in plates seeded with *Proteus vulgaris* and *Pseudomonas aeruginosa* when cultivated in the presence of *B. cereus*.

Preliminary work indicates that at least one inhibitory substance is present in the filtrate of proteose peptone broth cultures of *B. cereus*. The antagonistic effect of *B. cereus* and the isolation of the inhibitory substance seem worthy of further investigation, especially because of the inhibitory activity against the genera *Salmonella* and *Shigella*.

## ANTIBACTERIAL ACTION OF HEXENOLACTONE. James W. Bartholomew and Francis L. Hervey, Department of Bacteriology, University of Southern California.

Hexenolactone has been found to have bactericidal, bacteriostatic, and inhibitory action against such organisms as *Staphylococcus aureus*, *Bacillus subtilis*, *Serratia marcescens*, *Escherichia coli*, *Shigella paradysenteriae* var. *sonnei*, *Shigella paradysenteriae* Flexner V, *Salmonella paratyphi*, *Salmonella schottmuelleri*, *Proteus vulgaris*, and *Pseudomonas fluorescens*. Bactericidal concentrations ranged from 1:100 to 1:400; bacteriostatic concentrations ranged from 1:200 to 1:1,000; inhibitory concentrations ranged up to 1:3,200.

The gram-staining properties of the organisms did not correlate with the effective-

ness of the drug, and the presence of 10 per cent serum did not markedly reduce the antibacterial action. For some organisms, the higher dilutions resulted in slight stimulation of growth. In several instances in the presence of serum, 1:100 dilutions were much less effective than 1:200 or 1:400 dilutions.

The LD<sub>50</sub> for 13- to 15-gram white mice was approximately 5.2 milligrams, for a single dose injected into the peritoneum.

#### BACTERIOLOGY OF SCLEROMA. Robert E. Hoyt and Milton Gjølhaug Levine.

An organism has been isolated from the nose and throat of cases of scleroma (rhinoscleroma), which has the following characteristics: It is a gram-negative rod which forms large mucoid colonies on eosin-methylene-blue agar and nutrient agar. Acid is formed in glucose, maltose, mannitol, and occasionally in sucrose, but never in lactose. This organism is not found normally in the nose or throat and does not correspond to any other bacterium described in the bacteriological literature. It is similar to organisms previously described in scleroma by a few scattered workers. Etiological evidence published elsewhere by us indicates that this organism may rightly be called *Klebsiella rhinoscleromatis*.

#### THE NATURE, PROPERTIES, AND TOXICITY OF SUBTILIN, AND ITS CHEMOTHERAPEUTIC

EFFECT ON THE COURSE OF EXPERIMENTAL INFECTIONS IN ANIMALS. A. J. Salle and Gregory J. Jann, Department of Bacteriology, University of California, Los Angeles, California.

The antibacterial product, subtilin, obtained from the cells of a certain strain of *Bacillus subtilis*, was found to be active chiefly against gram-positive bacteria. Two notable exceptions were *Neisseria gonorrhoeae* and *Neisseria catarrhalis*, both gram-negative but also antagonized by the antibiotic. Acid-fast organisms, including *Mycobacterium tuberculosis*, were also found to be susceptible to the antibiotic.

The agent showed an extremely low toxicity to embryonic chick heart tissue fragments cultivated *in vitro*. Under the conditions of the test, subtilin was found to be approximately 20 times more toxic to *Staphylococcus aureus* than to chick heart tissue, a remarkably low figure for a chemotherapeutic agent.

Subtilin was shown to exert a powerful *in vivo* action on a number of bacterial infections in mice and guinea pigs. Animals infected with type III pneumococcus, *Bacillus anthracis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* were quickly and easily cured of the infections. Recovery from the infections was so spectacular that it was almost beyond belief. The antibiotic produced no observable toxic reactions in the animals.

### WASHINGTON BRANCH

WASHINGTON, D. C., MARCH 25, 1947

#### DEMONSTRATION OF AGGLUTINATION AND AN AGGLUTININ-"BLOCKING" PROPERTY IN SERA OF KNOWN CASES OF BRUCELLOSIS. J. J. Griffiths, U. S. Public Health Service, Biologics Control Laboratory, National Institute of Health, Bethesda, Maryland.

Immunological techniques useful in detecting sensitization to the Rh blood factor have been adapted to the examination of sera from individuals known to have had brucellosis. As in certain instances of Rh sensitization, sera of some brucellosis cases lack the ability to agglutinate *Brucella* organisms; they render the antigen insensi-

tive to the action of known agglutinins added to such serum-antigen mixtures. This agglutinin-"blocking" property of serum is present in certain sera to a much greater extent than in normal serum. Sera lacking agglutinins on routine tests may agglutinate *Brucella* strains when a normal serum is used in place of saline as a diluent. This appeared to be true in the test tube as well as on the warmed glass plate. These findings suggest that the use of such techniques may detect immunological responses in brucellosis and perhaps other diseases although the usual tests for diagnosis using saline diluent may be negative.

## EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND NINETY-THIRD MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY  
BUILDING, PHILADELPHIA, PENNSYLVANIA, MARCH 26, 1947

CONSTITUTIONAL FACTORS IN RESISTANCE TO INFECTION: THE EFFECT OF ESTROGEN ON THE PATHOGENESIS OF TUBERCULOSIS. *Maz B. Lurie, Samuel Abramson (U.S.P.H. Service), and Marvin J. Allison, The Henry Phipps Institute of the University of Pennsylvania, Philadelphia, Pennsylvania.*

Estrogen in large doses retarded the progress of tuberculosis at the site of inoculation in the skin and the dissemination of the disease in the internal organs as compared with the progress of the disease in rabbit litter mates of the same genetic constitution and of similar hereditary resistance to the infection. On the other hand, the periodic, intravenous injection of chorionic gonadotropin, which induced successive crops of corpora lutea, accelerated the progress of the disease in the majority of highly inbred litter mates. Physiologic quantities of progesterone and estradiol exerted no consistent effect on the process, nor did ovariectomy.

Estrogen suppressed tuberculin sensitivity of the skin not only in animals with

active disease but also in rabbits treated with heat-killed tubercle bacilli, but the sensitivity of the internal organs was not diminished. Since chorionic gonadotropin also reduced skin allergy, this effect of estrogen was not the significant factor in its retardation of the disease. Estrogen reduced the inflammatory irritability of the skin to unrelated noxious agents and markedly suppressed amyloid degeneration which is incidental to chronic tuberculosis. Ovariectomy, chorionic gonadotropin, and progesterone did not inhibit amyloidosis. Chronic estrogen treatment induced a lymphopenia and was associated with a reduction in the weight of the adrenals. There is some evidence that estrogen enhances the elimination of antibodies from their depots.

It is suggested that estrogen retards the tuberculous process by reducing the dissemination of the bacilli from the portal of entry, by sparing parenchymal degeneration, and by mediating the release of antibodies through the dissolution of lymphocytes.

## NEW YORK CITY BRANCH

COLLEGE OF THE CITY OF NEW YORK, NEW YORK, MARCH 27, 1947

COLIFORMS WITH COMPLETE SALMONELLA ANTIGENS, OR LACTOSE-FERMENTING SALMONELLAE? *Ivan Saphra and Erich Sligmann, Beth Israel Hospital, New York.*

In a previous communication the authors reported on "A coliform bacterium with the complete antigen of *Salmonella newington*." The organism isolated from a fatal meningitis in a 6-month-old baby grew on SS plates like a colon bacillus, fermented lactose, was H<sub>2</sub>S-negative, and had the antigenic formula of *Salmonella newington*. It split off a white growing variant, which also fermented lactose on subculture and produced H<sub>2</sub>S. Through papilla formation it gave rise again to the original coliform. Both types were serologically identical. This observation provoked a discussion

with two points of view: evolution of a *Salmonella* out of a normal *Escherichia coli*, or haphazard variation of a *Salmonella* resulting in the splitting off of lactose-fermenting substrains.

An almost identical phenomenon was observed recently. Again a culture with the antigenic pattern of *S. newington* was isolated, this time from the stool of a man ill with gastroenteritis. It grew on SS plates with the typical appearance of a *Salmonella*; on subculture, however, it fermented lactose with little gas and formed H<sub>2</sub>S abundantly. In further transplants the white colonies produced red papillae. These red mutants decomposed lactose rapidly with acid and much gas; they failed to produce H<sub>2</sub>S. So far this observation



parallels the older one. This time, however, a third variant was found, a typical *Salmonella*, lactose-negative for 3 weeks. All three variants, the coliform, the intermediate, the *Salmonella*, were serologically identical. Again it is impossible to ascertain whether *S. newington* was the end product of a variation or the source of a retrogressive variation. At any rate, the first finding is no more a unique curiosity; the repetition, again observed under natural conditions, perhaps indicates its general biologic importance.

CHEMICAL AND IMMUNOLOGIC STUDIES OF LOW RAGWEED POLLEN EXTRACT. *H. S. Baldwin, A. W. Moyer, and P. F. deGara*, Cornell University Medical College and New York Hospital, New York.

The immunologic activity of fractions of ragweed pollen extract was compared with that of standard pollen extract. Fraction B contained approximately 7 per cent nitrogen and 14 per cent carbohydrate; fraction D, approximately 5 per cent nitrogen and 58 per cent carbohydrate; and fraction S,

approximately 1.4 per cent nitrogen and 50 per cent carbohydrate and gave a negative ninhydrin test. Standard pollen extract and fractions B and D were precipitated by antiragweed serum. Standard pollen extract and fraction B were also precipitated by antifraction B serum. No precipitations were observed with antifraction D serum.

Sensitization of guinea pigs to standard extract was produced with each fraction. Sensitization to fraction B was not produced with fraction S. Sensitization to fraction D was produced with fraction D. No sensitization to fraction S could be produced. The threshold of sensitivity of untreated ragweed-sensitive persons to standard extract was lower than to the fractions.

The carbohydrate fraction of ragweed pollen extract is not an active antigen. The further the attempts to purify and fractionate the ragweed pollen extract were carried out, the less consistent were the immunologic reactions observed. Immunologic reactivity diminished with a decrease in the nitrogen content.

#### MICHIGAN BRANCH

ANN ARBOR, MICHIGAN, MARCH 27, 1947

ELECTRON MICROSCOPY AS APPLIED TO SOME BACTERIOLOGICAL PROBLEMS. *Ruth Lofgren*, Department of Bacteriology, University of Michigan, Ann Arbor, Michigan.

New interest in the cytology of microorganisms has resulted from the development of the electron microscope. The preparation of biological material presents many problems. Distilled water suspensions of bacteria from solid culture medium when dried on collodion-covered disks generally make good specimens. Because of the wide variations in the sources of organisms, techniques suitable for each type of material must be developed.

Vital processes cannot be observed, but consecutive preparations give similar information. Studies of bacteria may clearly demonstrate cell structures such as cell wall and flagella. Mechanical damage to cells may give additional information, the

fibrous cell wall and granular cytoplasm of spirochetes. The effects of treatment with immune serum, bacteriophage, chemicals, etc., can be observed in detail. Selective staining has shown promise in some cases. The shadow casting of films reveals surface structure or topography. By combining the various techniques, information can be obtained which can contribute much to our knowledge of microorganisms.

OBSERVATIONS ON PHAGOCYTOSIS WITH THE AID OF DARK-FIELD ILLUMINATION. *Donald J. Merchant and W. J. Nungester*, Department of Bacteriology, University of Michigan, Ann Arbor, Michigan.

With the aid of dark-field illumination the cytoplasmic granules of leucocytes were seen as highly refractive bodies exhibiting very rapid brownian motion. A layer of clear ectoplasm could be distinguished between the granular cytoplasm and the

cell wall, and a clearly demarcated nucleus was observed within the cytoplasmic mass.

True ameboid motion and phagocytosis were observed only in the presence of serum. In its absence a random pseudopod formation occurred as well as an infrequent phagocytosis due to chance contact. The addition of serum to an inactive suspension resulted in an increased brownian motion of the granules, accompanied by a corresponding increase in the activity of the cells. A

similar stimulation was observed when the electrolyte content of the suspending medium was lowered somewhat below physiological concentrations.

A suitable surface was necessary for the leucocytes to anchor on to form pseudopods. A carefully cleaned glass surface or fibrin strands proved satisfactory. Active cells could be obtained only from animals having a high ascorbic acid level.

### NORTHWEST BRANCH

UNIVERSITY OF WASHINGTON, SEATTLE, WASHINGTON, APRIL 5, 1947

A REINVESTIGATION OF BACTEREMIA IN PULMONARY TUBERCULOSIS. *W. F. Kirchheimer*, Department of Microbiology, University of Washington School of Medicine, Seattle.

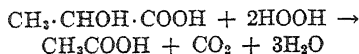
Blood samples were obtained from 15 patients with far-advanced pulmonary tuberculosis on three occasions at intervals of 2 weeks. Two samples were taken each time, one from the median cubital vein and one from the femoral artery. Each 5-ml sample was citrated, hemolyzed with distilled water, centrifuged vigorously, and the sediment suspended in distilled water. It was incubated 1 hour at 37 C to complete the hemolysis, centrifuged, and resuspended in physiological saline to give a volume of 2 ml.

This concentrate was evenly distributed into ten 2-ounce medicine bottles containing Petragani's medium and incubated at 37 C for 4 months. All of the 900 bottles inoculated were negative for tubercle bacilli. The insensitivity of the method used or the probable intermittent character of bacteremia in tuberculosis may account for the negative findings. A test of the method made with blood containing known numbers of tubercle bacilli indicated that at least 100,000 organisms per 5 ml of blood are necessary to initiate growth on the majority of the 10 bottles inoculated.

HYDROGEN PEROXIDE IN THE METABOLISM OF *LACTOBACILLUS BREVIS*. *H. C. Douglas*, Department of Microbiology, University of Washington, Seattle.

Suspensions of *Lactobacillus brevis* oxidize

glucose and lactate quantitatively to acetate and CO<sub>2</sub> without the accumulation of hydrogen peroxide. When suspensions are incubated anaerobically with lactate and hydrogen peroxide, lactate is oxidized and hydrogen peroxide is reduced according to the equation:



The suspensions have no action upon lactate alone anaerobically, nor upon hydrogen peroxide alone, aerobically or anaerobically. The oxidation of lactate by peroxide does not occur if the suspension is killed by heating. It is apparent that *L. brevis* possesses an enzymatic mechanism for activating hydrogen peroxide as an oxidizing agent, and by definition such an enzyme would be called a peroxidase. However, the reaction is not sensitive to cyanide and the usual tests for peroxidase are negative.

It seems probable that in the normal oxidative metabolism of *L. brevis* the hydrogen peroxide which theoretically should be formed as the first reduction product of oxygen is activated enzymatically as a hydrogen acceptor and reduced to water as rapidly as it is formed. Greisen and Gunsalus (*J. Bact.*, 45, 16) have reached similar conclusions concerning the metabolism of *Streptococcus mastitidis*.

DISCOVERY OF A BACTERIOPHAGE FOR *MYCOBACTERIUM SMEGMATIS*. *Grace M. Gardner and Russell S. Weiser*, Depart-

ment of Microbiology, School of Medicine, University of Washington, Seattle.

During investigations on the isolation of bacteria antagonistic to the mycobacteria a bacteriophage for *Mycobacterium smegmatis* was encountered. The enrichment method of Dubos was applied to six samples of moist leaf compost containing calcium carbonate. They were incubated at 37°C for 8 months and treated semiweekly with a heavy, washed suspension of *Mycobacterium smegmatis*. After 3 months tests for antagonists were begun by fixation-plating with 1 per cent glycerol agar heavily inoculated with *Mycobacterium smegmatis*. Plates from two composts contained

smooth-edged plaques displaying a halo of partial lysis about a central clear zone of complete lysis.

Serial Berkefeld filtrates of plaque material contained the bacteriophage in a concentration of 300 billion per ml. The bacteriophage proved inactive for *Mycobacterium phlei* and a second strain of *Mycobacterium smegmatis*. Its thermal death point was between 72°C and 75°C. It preserved well in 50 per cent glycerol, and by lyophilization.

The lack of reports of bacteriophages for the mycobacteria indicates that they may be scarce. The present isolation may have succeeded because of the enrichment procedure employed.

## OHIO BRANCH

OXFORD, OHIO, APRIL 12, 1947

STUDIES IN HODGKIN'S SYNDROME. VII. CYTOPATHOLOGIC RESPONSES OF TISSUE CULTURES INOCULATED WITH AGENTS FROM HODGKIN'S DISEASE AND LYMPHOMATOSIS. Jackson W. Riddle, Miriam S. Flower, Margaret S. Reiman, and Herman A. Hoster, Departments of Bacteriology and Medical Research, Ohio State University, Columbus.

Many microorganisms have been implicated, but none have been proved, to be the etiologic agent of Hodgkin's disease. Grand (1944) described the presence of giant cells and intracytoplasmic inclusion bodies in Hodgkin's tissues cultured *in vitro*, nourished with chicken embryo extract and plasma, and stained with Seller's stain. Hoster, Riddle, Flower, and Reiman (1947) described a similar cytopathologic phenomenon which appears occasionally in cultures of supposedly normal chick embryo spleen, and which occurs consistently in homologous guinea pig fetal spleen cultures inoculated with cell-free extracts or ultracentrifuge preparations of Hodgkin's and lymphomatosis tissues and body fluids.

Descriptions were presented of these specific cytopathologic responses: Hodgkin's-like cells, fuchsinophilic intracytoplasmic inclusion bodies, cyto stimulation, degeneration, and alterations in the pro-

portions of the various cell types. Control preparations, either inoculated, or uninoculated with preparations obtained from human sources other than Hodgkin's disease, have failed to produce these specific cytopathologic alterations.

Preliminary experiments suggest that rabbit macrophage cultures may be used as substrate for the production of these inclusion bodies.

A MAXIMUM DILUTION METHOD FOR THE QUANTITATIVE DETERMINATION OF PNEUMOCOCCAL POLYSACCHARIDE IN SOLUTION. Curtis Sandage and Orton K. Stark, Department of Botany and Bacteriology, Miami University, Oxford.

The most accurate method for the quantitative determination of pneumococcal polysaccharide has been based on estimation of specifically precipitable antibody nitrogen. This procedure is subject to all of the limitations and difficulties of interpretation encountered in precipitin tests. In a study involving the preparation of pneumococcal polysaccharide it became evident that a more sensitive and more easily interpreted method might be of value.

The method evolved depends on the sensitivity of mouse response to minute quantities of antigenically active polysac-

charide. By proper standardization of procedures, pneumococcal polysaccharide in solution and in body fluids can be determined quantitatively in amounts not detectable by the usual precipitin tests. For example, 0.00001 mg of purified SI (capsular polysaccharide) were detected by this method, although five times this amount was required to produce a positive precipitin test.

This method has been used to estimate the SI content of crude preparations, with highly consistent results. This indicates that it is applicable to the quantitative determination of both crude and purified SI in solution.

SOME PROPERTIES OF A MUCOPOLYSACCHARIDE ISOLATED FROM A STRAIN OF *CLOSTRIDIUM PERFRINGENS*. *Alfred A. Tytell, Milan A. Logan, and Alice G. Tytell*, Department of Biological Chemistry, University of Cincinnati, Cincinnati.

A highly viscous, alcohol-insoluble polysaccharide has been isolated from cultures of *Clostridium perfringens* (F5022, Lister Institute). The substance is soluble in water and shows characteristic carbohydrate reactions. It contains no phosphorus and less than 0.1 per cent nitrogen. Spectrophotometric studies of the reactions with orcinol, phloroglucinol, and diphenylamine indicate the presence of pentoses. Reaction with naphthoresorcinol and carbazole indicate the absence of uronic acids. Quantitative determination of the hydrochloric acid degradation products indicate 80 per cent recovery as furfural. These results predict that the substance may possibly be a pentosan. Preliminary studies indicate that the substance is not antigenic.

IMMUNIZATION OF MICE WITH DYSENTERY ANTIGEN ADMINISTERED BY GAVAGE OR BY VOLUNTARY DRINKING. *Merlin L. Cooper and Helen M. Keller*, The Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati.

In the course of attempts to infect white mice by gavage with living *Shigella sonnei*,

we have observed the development of immunity. A high degree of immunity resulted if the mice were given large numbers of bacteria as antigen. Less immunity followed administration of fewer bacteria as antigen.

Six doses of 49 billion living *Shigella sonnei* administered by gavage, over 4 consecutive days, stimulated complete immunity against intraperitoneal injection of 16 to 160,000 MLD of homologous organisms suspended in sterile mucin.

Three to 8 daily doses of 1.4 billion living *Shigella sonnei*, or 1.1 billion killed *Shigella sonnei*, given by gavage, stimulated a significant degree of immunity in the mice when challenged 7 days later with intraperitoneal injections of 0.5 to 1,280 MDL of the homologous organisms. Three daily doses stimulated sufficient active immunity to protect at least 50 per cent of the mice and 8 daily doses afforded protection to at least 80 per cent of the mice. The titer of the immunity increased with increasing number of daily doses of antigen. There was no significant difference in the immunizing effects of living or killed organisms.

Mice which drank a killed broth culture of *Shigella sonnei* over a period of 21 days were immune against 2 to 2,048 MLD of the homologous organisms when injected intraperitoneally. These mice consumed an average of 7.5 billion killed *Shigella sonnei* per day.

BIOCHEMICAL STUDIES OF A SOFT X-RAY MUTANT OF *ASPERGILLUS NIGER* VAN TIEGHAM. *Violet M. Diller, Alfred A. Tytell, and H. Kersten*, Departments of Physics and Biological Chemistry, University of Cincinnati, Cincinnati.

Nutrition studies have been made on a soft X-ray mutant of *Aspergillus niger* van Tiegham. The mutant is stimulated 240 per cent by biotin as against 70 per cent for the normal. Hypoxanthine, inositol, and *p*-aminobenzoic acid stimulate the mutant 140 to 200 per cent as against 10 to 50 per cent for the control. Pyridoxine, pimelic acid, riboflavin, thymine, guanine, niacin, and cytosine stimulated the mutant 40 to 75 per cent but did not stimulate the normal.

Analyses of the lanthanum-precipitable fractions indicated that the mutant nucleic acid content was at least 25 per cent lower (on a dry weight basis) than the normal. This was confirmed by spectrophotometric data.

**ACCELERATED PRODUCTION OF POLIOMYELITIS.** John A. Toomey, William S. Takacs, and P. P. Pirone, Division of Contagious Diseases, City Hospital, Cleveland.

Succinic acid, chlorophyll, and a synthetic preparation—succinonitrile—when injected into cotton rats (0.1 ml I.C.; 1 ml S.Q.; and 2 ml I.P.) in concentrations of 0.03 per cent, 0.06 per cent, and 0.027 per cent, respectively, in distilled water, conditioned cotton rats so that, when 2 to 3 weeks later they were injected intracerebrally with 0.1 ml of a 10 per cent saline suspension of Flexner's cotton-rat-adapted strain, there was an acceleration in the production of poliomyelitis. Chlorophyll alone did not cause such an acceleration.

**THE EFFECTS OF MALIC AND MALONIC ACIDS ON METHYLENE BLUE REDUCTION BY BACTERIA.** Chester I. Randles and Jorgen M. Birkeland, Department of Bacteriology, Ohio State University, Columbus.

Attempts to demonstrate malonate inhibition of methylene blue reduction by *Escherichia coli* with succinate as the hydrogen donor were unsuccessful. Concentrations of malonate as high as 100 times that of the succinate were tried. With *Pseudomonas aeruginosa*, however, inhibition is readily demonstrable. Equal concentrations of malonate and succinate (0.019 M) result in reduction times from 2.5 to 3 times longer than those with succinate alone.

With certain concentrations of substrate, the reduction of methylene blue with fumarate is more rapid than with malate. This is demonstrable with *E. coli* grown on glucose synthetic media but not with *P. aeruginosa*.

*P. aeruginosa* shows much greater dehydrogenase activity for acetate, succinate, fumarate, and malate than does *E. coli* when both are grown on glucose synthetic media. However, when acetate is substituted for glucose, the activity of *E. coli* is increased at least tenfold and is comparable to that of *P. aeruginosa*. The high activity seems to be associated with aerobic growth and indicates the intermediation of the C<sub>4</sub> dicarboxylic acids in acetate oxidation.

## EASTERN NEW YORK BRANCH

TROY, NEW YORK, APRIL 18, 1947

**A STUDY OF HEMOPHILUS PERTUSSIS BY MEANS OF THE ELECTRON MICROSCOPE.** Julia M. Coffey and Sophia M. Cohen, Division of Laboratories and Research, New York State Department of Health, Albany.

A preliminary comparative study was made of *Hemophilus pertussis*, directed particularly to the effect of age and medium on the cell and to a possible correlation between cellular structure and antigenicity. Four phase I strains were morphologically similar but different from both a pertussis strain not in phase I and two parapertussis strains. Electron micrographs differentiated an outer membrane in all strains. In 1-day cultures on potato-infusion rabbit-blood agar, the cytoplasm of phase I strains

was of relatively uniform density; in 2- and 3-day cultures, two types of opacity were observed—diffuse areas near the ends and clearly circumscribed spherical granules frequently centrally located. Cultures in blood-free semisynthetic fluid medium had similar morphologic characters but the granules were rare and less distinct. The cytoplasm of the pertussis strain not in phase I contained one or more irregularly shaped semitransparent areas; the granules were rare. *Hemophilus parapertussis* from solid medium resembled *H. pertussis* phase I except that the granules were commonly observed in 1-day cultures.

**PROPAGATION OF LYMPHOCYTIC CHORIO-MENINGITIS VIRUS IN EMBRYONATED**

**HENS' EGGS.** *Lisbeth M. Kraft and Irving Gordon*, Division of Laboratories and Research, New York State Department of Health, Albany.

The virus of lymphocytic choriomenigitis has been grown by others on the chorio-allantois and in the yolk sac of embryonated hens' eggs. This study was undertaken to determine the optimal route and incubation period for attaining high virus titer, particularly in the extraembryonic fluids, for the production of complement-fixing antigen.

Eggs were inoculated via the yolk sac, chorioallantoic membrane, and allantoic sac, and various incubation periods were arbitrarily chosen. Yolk sac and chorioallantoic membrane of the respective series and allantoic fluid of the three series were harvested and tested intracerebrally in mice. The lethal titer of serial 10- or 100-fold dilutions of the first and fourth passage material was determined. The results indicate that allantoic fluid is not a rich

source of the virus but that the tissues tested contain considerable amounts (titers of  $10^{-7}$ ).

Further studies are in progress using the amniotic route as well as the yolk sac and chorioallantoic routes.

**EFFECT OF SOIL ACTINOMYCETES AND pH ON THE MM STRAIN OF POLIOMYELITIS VIRUS.** *Albert Schatz*, Division of Laboratories and Research, New York State Department of Health, Albany.

Culture filtrates of soil actinomycetes antagonistic to bacteriophages were tested for antibiotic activity on the MM strain of poliomyelitis virus. Inoculations were made intraperitoneally in mice. Under the conditions of the experiment, none of 59 preparations exerted any definite antagonistic effect on the virus. Results under the conditions employed indicated the necessity for careful control of the pH of the test material.

## WASHINGTON BRANCH

ONE HUNDRED AND FIFTY-SIXTH MEETING, COLLEGE PARK, MARYLAND,

APRIL 22, 1947

**THE EFFECTS OF LACTOBACILLI ON THE QUALITY OF CHEDDAR CHEESE MADE FROM PASTEURIZED MILK.** *Ralph P. Tittsler, George P. Sanders, Homer E. Walter, Donna S. Geib, Oscar S. Sager, and Harry R. Lochry*, Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

Cheddar cheese was made from milk of good quality, which was pasteurized and divided equally into two lots. Lactic starter was added to one lot. Lactic starter and a supplemental starter containing lactobacilli were added to the other lot. The bacterial flora and chemical changes in the cheese were determined at 1 day, 2 weeks, and 1, 2, 3, 4, and 6 months.

*Lactobacillus casei* (three types), *L. arabinosus*, *L. pentosus*, *L. fermenti*, and *L. plantarum* (including several types isolated from Cheddar cheese made from raw milk) grew rapidly in the cheese. The maximum

numbers of lactobacilli, from 50 to 500 millions per gram, were present at from 2 to 12 weeks, depending on the species and numbers added and on the temperature (50 or 60 F) of ripening the cheese.

*L. bulgaricus*, *L. helveticus*, *L. lactis*, and *L. acidophilus*, added to the milk in relatively large numbers, were not detected in cheese ripened for 2 weeks or more at either 50 or 60 F. The grades of the cheese were the same as those of the corresponding controls.

*L. fermenti* produced gas and a decidedly objectionable flavor in the cheese. *L. casei* increased the acidity in the cheese but did not increase proteolysis. It increased the development of flavor but, with prolonged curing, it usually produced an acid flavor and "short" body. Some strains of *L. arabinosus*, *L. pentosus*, and *L. plantarum* increased the development of flavor and did not increase the acidity to an objectionable extent. Other strains had little if any effect on the quality of the cheese.

## EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND NINETY-FOURTH MEETING, PHILADELPHIA, PENNSYLVANIA,  
APRIL 22, 1947

**AIR SAMPLING PERFORMANCE.** *Cretyl Crumb and W. F. Wells*, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

In studies of airborne infection, pathogens on droplet nuclei are of greater hygienic significance than ubiquitous saprophytes on dust. Therefore a standard instrument capable of efficiently sampling droplet nuclei in air is essential.

The air centrifuge at  $\frac{1}{2}$  cfm, at 1 cfm, slit sampler, aeroscope, sieve, and funnel recovered from 1 cubic foot of air the following number of fine droplet nuclei per fine droplet nucleus settling on a petri plate in one minute: 841.5, 277.5, 385.5, 106.5, 27.8, and 1.5, respectively. Against coarse nuclei the counts were 83.3, 49.5, 39.8, 23.3, 23.3, and 2.3, respectively.

These results were confirmed by operating instruments in tandem. The slit sampler (following aeroscope), and centrifuge (following sieve, or funnel) collected, respectively, 1.7, 47.6, and 160.1 times as many fine nuclei as were removed from this air by the preceding instruments. For coarse nuclei the figures are 0.4, 0.7, and 13.3.

Evidently the efficiency of sampling instruments increases with increase in particle size; and for sampling dust particles, which are much larger than these coarse nuclei, even the settling plate is satisfactory. But for recovering fine droplet nuclei, one of the more efficient air samplers, such as the centrifuge or the slit sampler, is indicated.

**EPIDEMIC OF INFLUENZA A AMONG A RECENTLY VACCINATED POPULATION: ISOLATION OF A NEW STRAIN OF INFLUENZA A VIRUS.** *M. Michael Sigel, Frank W. Shaffer, and Werner Henle*, The Children's Hospital, Philadelphia, Pennsylvania.

In March, 1947, an outbreak of upper respiratory infection occurred in a school in New Jersey. Practically all of the students had been vaccinated against epidemic influenza early in December, 1946. The epidemic was diagnosed as influenza A both by etiological and serological tests.

The virus L<sub>247</sub> was isolated from pooled throat washings by amniotic inoculation of chick embryos. It proved to be antigenically distinct from the PR8 and Weiss strains of influenza A incorporated in the vaccine. Most of the individuals possessed high titers to the PR8 strain of influenza A in the acute serum specimen, indicating that the vaccine was of satisfactory potency, a fact which has been confirmed by mouse vaccination tests. The acute sera showed low titers to the L<sub>247</sub> virus.

Comparative studies on acute and convalescent sera by the inhibition of hemagglutination gave significant rises in all convalescent sera when tested with the L<sub>247</sub> strain. The complement-fixation test with PR8 virus was as satisfactory and almost as sensitive as the inhibition of hemagglutination with the homologous virus. Both tests were far superior to the inhibition of hemagglutination with PR8 or Weiss viruses which, in a number of individuals, failed to demonstrate rises in titer.

**A SERIOLOGICAL TYPE OF PARACOLON AS A PROBABLE CAUSE OF AN EPIDEMIC OF GASTRO-ENTERITIS.** *T. F. McNair Scott, Lewis L. Coriell, H. Davis, and Ben H. Boltjes*, Children's Hospital, Philadelphia, Pennsylvania.

At the end of a short, mild, but extensive, outbreak of gastro-enteritis at a children's camp, a bacteriological survey was made of 43 patients and 10 food handlers. Two weeks after the last case, 26 of the previous patients, 33 controls, and 9 food handlers were again studied. Rectal swabs revealed *Paracolobacterium* organisms in 28 per cent in the first survey and 15 per cent in the second. Biochemically, 7 were *P. intermedius*, 4 *P. aerogenoides* (1 somewhat different), and 1 was different, in the first survey; in the second, 10 were different, resembling atypical *Paracolobacterium* or, possibly, *Proteus* organisms. An antiserum to one formolized *P. aerogenoides* agglutinated the 11 similar *Paracolobacterium* organisms from the first survey, but none of those from

the second. Pooled 32011 antiserum behaved similarly. Antisera to 2 other first survey organisms and cross-absorption tests revealed 4 antigenic subgroups with interlocking antigens: one in group I, with wide antigenic crossing with the other groups; 2 in group II, 7 in group III, and 1 in group IV. Representatives of the first 3 groups were agglutinated by patients' convalescent sera, but also by control sera. Further studies revealed such antibodies present in well babies and nondiarrhoeal patients in a different community, after the age of 6 months, but not before. Since antibodies from camp sera were easily absorbed by representative organisms, they possibly resulted from a wide exposure to these organisms, rather than being natural antibodies. The serological studies were conducted with O antigens. Epidemiological evidence implicated *Paracolobactrum*; 60 per cent of the organisms isolated formed a serological group and another 25 per cent were very closely related.

STUDIES ON THE COMPLEMENT FIXATION ANTIGENS IN MUMPS. *Gertrude Henle, Werner Henle, and Susanna Harris*, Children's Hospital, Philadelphia, Pennsylvania.

It has been demonstrated that there exist

at least two serologically distinct antigens in chick embryos infected with mumps virus. One is intimately linked with the infectious agent; the other is smaller in size and may be termed a "soluble" antigen. Upon allantoic injection the virus-bound antigen is found mainly in the allantoic fluid, the soluble antigen largely in the allantoic membrane. The virus-bound antigen is sedimented by high-speed centrifugation at 20,000 rpm for 20 minutes, and the soluble antigen, for the greater part, at 30,000 rpm for 60 minutes. These two antigens can be differentiated by absorption of convalescent serum. Absorption with the soluble antigen leaves the antibodies to the virus-bound antigen intact, and absorption with virus particles does not markedly decrease the antibodies to the soluble antigen.

Upon studying sera of patients during and after an attack of mumps, and sera of people with a past history, a variable response to the two antigens has been observed. These observations suggest that for the determination of susceptibility of an individual, virus-bound antigen should be employed; for the demonstration of either recent infection or contact with mumps virus the soluble antigen, and, for diagnostic purposes, both antigens will give reliable information.

### CONNECTICUT VALLEY BRANCH

STORRS, CONNECTICUT, APRIL 24, 1947

THE PROPHYLAXIS OF RHEUMATIC FEVER WITH SULFONAMIDES. *Nelson K. Ordway*, Yale School of Medicine, New Haven, Connecticut.

Twelve reports have appeared in the past eight years concerning the efficacy of sulfonamides, chiefly sulfanilamide, in preventing recurrences of rheumatic fever in individuals who have had previous attacks. When severe statistical criteria are employed, it is found that only two of the papers report significant results. These two studies are, however, highly significant; and if less rigid criteria are employed, four of the other studies assume significance. Though the remaining reports fail to achieve statistical significance, they indicate without exception a reduced

incidence of rheumatic fever in individuals receiving sulfonamide prophylaxis. The lowered incidence of rheumatic fever was paralleled by a reduction in the incidence of disease due to group A hemolytic streptococci and in the number of carriers of this organism. These studies are of importance, not only to the clinician in making available a tool for the control of rheumatic fever, but also to the bacteriologist in providing additional evidence in support of the now generally accepted thesis that rheumatic fever represents an allergic response to infection with the hemolytic streptococcus.

A SIMPLE MEDIUM FOR GROWTH OF TUBERCLE BACILLI. *Donal L. Dunphy and*



Mildred D. Fousek, Yale University School of Medicine, New Haven, Connecticut.

This preliminary study suggests that a medium consisting of lysed blood and glycerol supports the growth of virulent tubercle bacilli. Growth is apparently more rapid than that occurring on Petragani's medium. It would seem that the amount of growth and the time requirement are in direct relation to the number of tubercle bacilli in the inoculum. If this medium continues to give reliable results, its simplicity of preparation and availability are assets for its use in the diagnostic laboratory. The problem of contamination can be eliminated from culture material such as sputums and gastric washings by digestion with 6 per cent sulfuric acid.

#### THE RELATIONSHIP BETWEEN pH TOLERANCE AND VIRULENCE OF BACTERIA.

J. M. Leise, Department of Bacteriology, Yale University School of Medicine, New Haven, Connecticut.

Virulence and pH tolerance (the ability to grow in alkaline broth) were found to be related when virulent and avirulent strains of *Shigella* and *Bacillus anthracis* were studied. The virulent bacteria were able to grow in alkaline broth with smaller inocula than the related avirulent bacteria. Differentiation occurred at pH 8.65 to 8.75 with the *Shigella* strains, and at pH 9.1 to 9.35 with the *B. anthracis* strains. The virulent strains also grew better than the avirulent in human and in horse serum. These results were thought to be due to the presence of proteolytic enzymes which are more effective in alkaline solution in the virulent than in the avirulent bacteria, for the pH tolerance of an avirulent *B. anthracis* strain was increased by adding trypsin to alkaline broth. Also, filtrates of broth cultures of virulent bacteria showed more proteolytic enzyme activity than did filtrates of the avirulent bacteria.

It was postulated that the proteolytic enzymes are associated with virulence by being related to the invasiveness (ability to grow in the body) but not to the toxicity of the organism.

#### EXPERIMENTAL INFECTION OF FLIES WITH

HUMAN POLIOMYELITIS VIRUS. Joseph L. Melnick and Lawrence R. Penner, Section of Preventive Medicine, Yale University School of Medicine, New Haven.

Nonbiting flies at epidemics of poliomyelitis have been found to harbor the virus of this disease regardless of whether they have been collected at rural, suburban, or urban areas. It is important to answer the question of the survival of the virus in the fly, especially as it pertains to possible multiplication in this host. It would appear that one should test fly species with feeding habits that make them most likely to be contaminated with virus in nature and that one should use strains of virus which appear in nature.

Human poliomyelitis virus, as naturally present in stools of poliomyelitic patients, has been fed to blowflies, *Phormia regina*. After this feeding, virus was found in the flies for 2 weeks, and in their excreta for 3 weeks.

Murine-adapted strains (Lansing and Y-SK) of poliomyelitis virus and Theiler's TO strain of spontaneous encephalomyelitis of mice behave like biologically inert carmine in flies (*Phormia regina*, *Phaenicia sericata*, and *Sarcophaga bullata*). Following their ingestion by flies they may be found in gradually decreasing quantities for a period of 5 days.

#### DETERMINATION OF BACTERIAL SENSITIVITY TO STREPTOMYCIN IN THE SMALL HOSPITAL LABORATORY. Kenneth N. Atkins, and Eleanor Hoag, Department of Bacteriology, Dartmouth Medical School, Hanover, New Hampshire.

Parallel experiments with the army dilution method and paper disks containing 5, 10, 25, 50, and 100 units of streptomycin placed on surface-inoculated blood agar plates showed comparable results. The disk method appears to be preferable for routine tests because of its simplicity.

Of the organisms tested, *Pseudomonas aeruginosa* is unique in that by the disk method hemolysis is inhibited by the 10-unit disk though the growth is inhibited only by the 50- and 100-unit disks. This observation may be a clue to the mechanism of hemolysis by this organism.

## KENTUCKY-TENNESSEE BRANCH

BOWLING GREEN, KENTUCKY, APRIL 26, 1947

ALCOHOLIC FERMENTATION UNDER REDUCED PRESSURE. *M. C. Brockmann*, Joseph E. Seagram and Sons, Inc., Louisville, Kentucky, and *T. J. B. Stier*, Department of Physiology, Indiana University, Bloomington, Indiana.

After inoculation with a distillery type yeast, a glucose yeast extract  $\text{KH}_2\text{PO}_4$  medium was maintained at 30 C under an absolute pressure close to the vapor pressure of water and at the same time sparged with water vapor. Control cultures, which were held at atmospheric pressure, were flushed with tank  $\text{CO}_2$  at the time of inoculation in order to reduce the oxygen tension of the medium to a level comparable to that in the low pressure cultures.

The pressure differences did not have a marked influence either on the rate of glucose utilization or on the ultimate yeast population. Under reduced pressure the concentration of alcohol in the medium never exceeded 0.65 g per 100 ml, whereas free acetaldehyde was depressed to almost one-half the concentration found in comparable control samples. In low pressure cultures 5.5 to 6.0 g of glycerol were formed per 100 g of glucose metabolized; on the same basis control cultures produced 3.0 to 3.5 g. With each type of culture, glycerol formation was a linear function of glucose utilization throughout the greater part of the observation period. However, under low pressure the output of glycerol per unit of yeast population per hour fell in the latter part of the observation period to approximately the same level as the control.

THE OXIDATION OF CARBOHYDRATES BY A SURFACE STRAIN OF *PENICILLIUM NOTATUM*. *Frederick T. Wolf*, Department of Biology, Vanderbilt University, Nashville, Tennessee.

This study is concerned with measurements of the oxygen consumption of a surface strain of *Penicillium notatum* (NRRL 1249), using the Fenn differential respirometer. The  $\text{QO}_2$  of *P. notatum*, as measured in the lactose corn steep medium in which the fungus was grown, varies with the age

of the culture, increasing to a value above 16  $\text{mm}^3$  per hr per mg at 3 to 4 days, and decreasing rapidly thereafter.

Glucose, galactose, mannose, maltose, and cellobiose are rapidly oxidized by *P. notatum*. Glycerol, calcium lactate, arabinose, xylose, rhamnose, fructose, sucrose, lactose, dextrin, mannitol, sorbitol, dulcitol, and adonitol are more slowly oxidized. Trehalose and soluble starch were not oxidized, under the conditions employed, by this strain of *P. notatum*. The significance of the findings in relation to commercial penicillin production was discussed.

A SURVEY OF THE POTABILITY OF WELL WATERS IN CENTRAL KENTUCKY. *Rafael A. Cartin* and *R. H. Weaver*, Department of Bacteriology, University of Kentucky, Lexington, Kentucky.

Water samples have been examined from 73 wells in central Kentucky, representing an area of four counties. Of these, 62 yielded coliform organisms. Fifty of the wells could be classified as heavily polluted, as judged by the number of coliforms in the samples. Of the 11 samples that did not yield coliforms, several gave relatively high 37 C standard plate counts. This may be interpreted as indicating potential danger.

Central Kentucky is a limestone region. The relative lack of potable water supplies in this region confirms the common finding that strata of limestone are poor filtering agents for ground water supplies. The wells that were included in the study varied from 25 to over 2,000 feet in depth and were located in various strata of limestone. Except for one 2,000-foot well, no correlation could be found between the depths of the wells or the strata in which they were located and the potability of the waters. The soil type that overlaid the water-bearing strata also did not appear to be a significant factor.

A RAPID METHOD FOR THE DETECTION OF BACTERIAL CONTAMINATION IN SUBMERGED MOLD CULTURES. *W. Schneider*,

*S. L. Adams, and W. H. Stark, Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.*

The rapid detection of bacterial contamination is of great importance in submerged mold amylase propagation. Amylase yields are reduced if contaminating bacteria are present. The use of solid or semisolid media for detecting contaminants is unsatisfactory because mold growth masks bacterial colonies and the granular nature of the inoculum makes the detection of pin point colonies difficult. A turbidimetric method was developed after noting that the mold, *Aspergillus niger*, produced

a pellicle but no turbidity in an enriched yeast extract glucose peptone medium. The contaminating organisms, however, produce a marked turbidity in the medium in 24 hours or less.

This method has been used with a high degree of success in detecting the presence of contaminating bacteria in both laboratory and pilot plant submerged mold amylase propagation. In most cases, results can be obtained in 6 to 18 hours, and in no instance has it taken more than 24 hours to detect easily the presence of contaminating bacteria.

#### TEXAS BRANCH

AUSTIN, TEXAS, APRIL 26, 1947

THE USE OF BEEF SERUM AS A DILUENT FOR CHICK MEMBRANE SMALLPOX VACCINE. *Patti Crain, Biologies Division, Texas State Health Department, Austin, Texas.*

The one undesirable aspect of chick membrane smallpox vaccine as produced by this laboratory since 1939 has been its lack of thermostability. Following reports by Buddingh and others on the protective effect of inactivated serum, lots of vaccine diluted with inactivated beef serum, inactivated beef serum with one unit of penicillin per ml, equal parts beef serum and glycerol, glycerol saline, and a control lot of calf lymph were compared. These vaccines, in capillary tubes, were stored at 37 C, room, and refrigerator temperatures, and were tested at intervals on rabbits by two series of intracutaneous titrations, by vaccinations, and by Leake and Force titrations. The lots of vaccine diluted with inactivated beef serum consistently retained activity longer.

After 18 months' use, a comparison between it and the old glycerol saline vaccine, on the basis of 32,000 reports from health officers throughout the state, shows that the old vaccine was giving an average of 76.45 per cent "takes," whereas the new has given 94.66 per cent "takes."

AGAINST THE BACILLI OF HUMAN TUBERCULOSIS. *Edwin A. Johnson and Kenneth L. Burdon, Department of Bacteriology and Immunology, Baylor University College of Medicine, Houston, Texas.*

A contaminant on a Sabouraud's agar plate was found to be inhibiting strongly the surrounding growth. On isolation this organism proved to be a moldlike actinomycete of unusual properties. It showed a sharply limited pH growth range, failing to multiply at pH 7.0 or above. Simultaneous inoculation of slanted media with various test organisms revealed a marked antagonism against several of the gram-positive and gram-negative pathogenic bacteria, including mycobacteria, yeast, and molds.

Filtrates of pure cultures in tryptone starch broth, and also the aqueous solutions ("mycomycin") obtained after ether or amyl acetate extraction, had a similar activity. The best extracts to date have completely prevented growth of the routine test organism (*Bacillus subtilis*) in dilutions of 1:7,500, and have stopped the growth of virulent human tubercle bacilli in approximately a 1:5,000 dilution. The presence of serum did not greatly reduce the activity. However, erythrocytes showed a definite adsorption curve over a 24-hour period. Highly active concentrates are nontoxic for mice.

MYCOMYCIN—A NEW ANTIBIOTIC PRODUCED BY A MOLDLIKE ACTINOMYCETE ACTIVE

THE DESTRUCTION OF HYALURONIC ACID  
BY CAPSULATED GROUP A STREPTOCOCCI.

Robert M. Pike and Nadine Salem, Department of Bacteriology and Immunology, Southwestern Medical College, Dallas, Texas.

When mucoid strains of group A streptococci are grown in serum broth, hyaluronic acid accumulates in the culture fluid as the capsules disappear from the cells. After the maximum concentration of hyaluronic acid is reached, it remains constant during continued incubation in cultures of some strains, but in others it was found to disappear in from 1 to 7 days. This decrease in hyaluronic acid concentration appears to be due to an extracellular enzyme, since sterile filtrates show the same decrease as whole cultures, but filtrates heated at 60 C for 30 minutes retain their hyaluronic acid. The enzyme produced by one strain will also destroy hyaluronic acid produced by another strain. These observations indicate that capsulated group A streptococci, as well as the noncapsulated strains previously described by others, may produce hyaluronidase. The enzyme activity of capsulated strains, however, is relatively weak and highly variable. The relation of this enzyme to the disappearance of capsules from the cells and to phase variation is not yet apparent.

ALTERING DRUG RESISTANCE OF BACTERIA  
WITH BACTERIAL EXTRACTS. Orville Wyss, Department of Bacteriology, University of Texas, Austin, Texas.

Sterile purified nucleoprotein extracts were prepared from a drug-sensitive strain of *Escherichia coli* and a drug-resistant

strain derived from it. The addition of the nucleoprotein extract from the sensitive strain to a young growing culture of the resistant strain resulted in a culture in which the number of highly resistant organisms was reduced. Conversely, the addition of the extract from the resistant strain to a growing culture of the sensitive strain quantitatively increased the distribution of resistant forms in the resulting population. The nucleoprotein extracts were separated into the nucleic acid and protein components. The nucleic acid was the active fraction.

A FACTOR TOXIC TO BRUCELLA ABORTUS IN  
SOME LOTS OF TRYPTOSE. V. T. Schurhardt and L. J. Rode, The Brucellosis Research Project of the Clayton Foundation and The University of Texas, Austin, Texas.

Three of 7 lots of Difco tryptose tested showed the presence of a factor which specifically suppressed the growth of inocula of 5 strains of *Brucella abortus* containing up to a billion or more viable organisms. The factor was not active against strains of *Brucella melitensis*, *Brucella suis*, or six other bacterial species tested. The factor in 2 per cent tryptose was shown to be brucellacidal in 48 hours against inocula of 400 to 500 organisms per ml. The toxic factor in tryptose broth is neutralized by blood, serum, Difco agar, and aqueous extracts of a number of plant and animal tissues. This fact limits the practical significance of the toxicity factor, but we believe that the factor may possess considerable biological significance and that efforts to determine the chemical nature of the factor are justified.

# CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

## III. EFFECT ON REACTION TO THE GRAM STAIN IN STAPHYLOCOCCUS AUREUS<sup>1</sup>

JEAN DUFRENOY AND ROBERTSON PRATT<sup>2</sup>

*University of California College of Pharmacy, The Medical Center,  
San Francisco 22, California*

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It has been shown by application of appropriate reagents to standard agar penicillin assay plates after different periods of diffusion of penicillin that—SH groups and dienols are present outside the zones of inhibition, but are relatively scarce or are lacking inside the zones (Dufrenoy and Pratt, 1947). The results indicated that a threshold effect involving an—SH $\rightleftharpoons$ S—S equilibrium exists at the boundaries of the inhibition zones. Our conclusions, based on the macroscopic evidence obtained by developing assay plates with reagents for—SH groups and for dienols, emphasized the significance of the sulfhydryl radical as an important part of a redox system and the possible relation of sulfhydryl groups to the mechanism of action of penicillin on susceptible organisms. Our second paper (Pratt and Dufrenoy, 1947a) correlated the macroscopic patterns on the "developed" assay plates with cytochemical changes induced within the test organisms by a bacteriostatic concentration of penicillin.

In the present paper we will present cytochemical evidence that correlates the bacteriostatic activity of penicillin against *Staphylococcus aureus* with changes in reaction to the gram stain and with each of the three cell constituents cited by Henry, Stacey, and co-workers (1943, 1945, 1946), as playing an essential role in a positive reaction to the gram stain, viz., (1) nucleoproteins, (2) arginine, and (3)—SH groups.

Convergent lines of evidence obtained by the use of various histochemical and cytochemical techniques indicate that, previous to inhibition, cells of *S. aureus* exposed to bacteriostatic concentrations of penicillin go through a climacteric period of enhanced metabolism, during which they consume sulfhydryl compounds more rapidly than they can reconstitute them (Dufrenoy and Pratt, 1947). Thus a depletion of active components of aerobic respiratory systems ensues. This may be assumed to result in failure of the supply of energy required for active absorption of solutes (Pratt and Dufrenoy, 1947a). A similar correlation is suggested by the work of Gale and Taylor (1946), who showed that bacteriostatic concentrations of penicillin block the absorption of the essential metabolite, glutamine, by *S. aureus*. Their observation that the first physiologically evident effect of penicillin is inhibition of glutamine assimilation appears extremely significant, since glutamine is a component of glutathione, the activity of the —SH group of which is known to depend markedly on the vicinal NH groups.

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<sup>2</sup> With the laboratory assistance of Toinie Juntunen.

Therefore it seemed desirable to study the distribution of amino acids in the various regions of penicillin assay plates after different periods of exposure to penicillin. The work was limited somewhat in scope because, obviously, techniques that require the use of heat could not be used, since the agar would have melted, and reagents which promptly hydrolyze an agar base could not be used.

#### MATERIALS AND METHODS

The techniques employed in this work were similar to those reported previously (*loc. cit.*). The only changes were in the reagents that were used. These are described under the individual experiments. *Staphylococcus aureus* NRRL 313 was used throughout.

It should be understood that the phrase "16-hour plates" refers to penicillin assay plates treated as prescribed by the Food and Drug Administration for the standard cylinder plate assay (similar to the recommendation of Schmidt and Moyer, 1944). The phrase "3-hour plates" refers to plates that were seeded, then incubated for 3 hours before the addition of "penicylinders," and then were subjected to a second period of incubation, during which period penicillin was permitted to diffuse from the cylinders. The details of the preparation of such plates are given by Goyan, Dufrenoy, Strait, and Pratt (1947).

#### EXPERIMENTS AND RESULTS

*Development with reagents for amino acids.* (1) Sakaguchi test for arginine. It is stated that this reagent when prepared according to the directions of Glick and Fisher (1946) may be considered specific for the detection of arginine in living cells. When assay plates incubated with penicillin for 3 hours, as in the 3-hour cylinder plate assay method, are treated with the  $\alpha$ -naphthol, hypobromite, and urea reagent, a positive reaction (manifested by development of red color) occurs in the areas of noninhibition, whereas the areas of inhibited growth develop a very faint pink color.

(2) Alloxan test for  $\alpha$ -amino acids. When 3-hour plates are flooded with a 1 per cent solution of alloxan in alcohol, a strong positive reaction (manifested by development of a deep red color) promptly appears in the background of normal uninhibited growth, whereas a very weak reaction (faint pink color) develops in the areas of inhibited growth, owing to the staining of the original colonies developed during the period of preliminary incubation without penicillin. It should be pointed out that although the alloxan-positive reaction may be considered as indicating the presence of amino acids, it has also been suggested that such a reaction might also indicate the presence of —SH groups (Serra, 1946).

(3) Millon's reagent for tyrosine. The phenolic amino acid, tyrosine, and its phenolic derivatives are held to be responsible for the very sensitive Millon reaction (Everett, 1946, p. 374). When a drop of this reagent is allowed to spread at the margin of a zone of inhibition, on a standard 16-hour assay plate seeded with *S. aureus*, the actively growing microorganisms at the outer margin of the inhibition zone give a very strong positive reaction within a few minutes, so that the clear inhibition zones appear surrounded by transient vivid red rings.

It should be noted, however, that we do not claim specificity for tyrosine in the site of the positive Millon's reaction, especially since the region giving the strongest positive reaction with this test corresponds with that shown previously to be richest in phenolic compounds (Dufrenoy and Pratt, 1947).

*Sulfhydryl groups in the gram-positive complex.* In 1944 Bartholomew and Umbreit pointed to "the involvement of sulfhydryl groups in the over-all gram reaction" and in 1945 Henry, Stacey, and Teece reported that gram-positive organisms differ from gram-negative organisms in that on autolysis at pH 8 and 37 C the former release some gram-positive nucleoprotein, made up of nucleic acids and of a basic protein, which in its native reduced state involves —SH groups. Historically, it is interesting to note that Bach and Delétang (1931) and Delétang (1932) observed that organisms fixed in oxidizing killing fluids tend to lose their gram-positiveness, and that even earlier Deusen (1918, 1923) showed that gram-positives could be converted into gram-negatives by a number of methods. He concluded that the change was the result of chemical processes. These observations can now be interpreted in terms of oxidation of the sulfhydryl groups.

Our experimental results show that cells of *S. aureus* rapidly lose their gram-positive staining reaction when exposed to bacteriostatic concentrations of penicillin. In other words, within the inhibition zones on standard 16-hour plates, where no positive reaction for sulfhydryl groups can be obtained, cells of *S. aureus* are no longer gram-positive. Cells picked from the uninhibited background of such plates, however, show the usual range of reaction to the gram stain that may be expected from a culture of that age, i.e., actively growing typical gram-positive cells are coexistent with senescent cells which have more or less lost their gram-positive reaction. Within the inhibition zones, the only gram-positive cells are those from the few penicillin-fast organisms that are constantly encountered on assay plates. However, practically all of the cells which persist within the inhibition zones fully decolorize under the same treatment that preserves the stain in the majority of cells outside the zones. The background of uninhibited growth on 3-hour assay plates consists of actively growing colonies, composed almost exclusively of gram-positive cells.

Within the range of diffusion of bacteriostatic concentrations of penicillin, however, there can be observed all stages of loss of gram-positiveness. It is notable that cells affected by penicillin, as they are in the process of division, swell into "diplococcuslike" units, and the gram-positive material appears as inclusions in two sharply defined regions, one toward each pole. Each of these gram-positive regions is homologous to the portions of the cells previously described as staining vitally with neutral red under a comparable stage of inhibition by bacteriostatic concentrations of penicillin (Pratt and Dufrenoy, 1947a). Those bodies have a strong affinity for various basic dyes, such as methyl green, and may be supposed to contain, besides the phenolic compounds previously alluded to, some nucleic derivatives. The next section, therefore, pertains to tests designed to reveal the distribution of nucleic acid derivatives in the areas of normal uninhibited growth and of inhibited growth on the test plates.

*Development with reagents for nucleic acid.* The following dyes have been recommended as reagents for the detection of nucleic acid: methylene blue, Nile blue, toluidine blue, bromocresol purple, methylene green, malachite green, and safranin O. When the 3-hour assay plates are flooded with an aqueous solution (5 mg per L) of any of these dyes, a sharp definition of the inhibition zones is obtained. These reagents agree in locating the boundary of the zone at the same distance (within the limits of experimental error) around a cylinder from which a given concentration of penicillin has been permitted to diffuse (Pratt and Dufrenoy, 1947b).

#### DISCUSSION AND CONCLUSIONS

The data obtained by the adaptation of different histochemical and cytochemical staining techniques to penicillin assay plates are recorded in this paper and its two predecessors in the series. The present discussion will embody the results of all three papers and is intended as a résumé and summary of our work to date on the subject.

A penicillin assay plate, with its zones of inhibited and of normal uninhibited growth of the test organisms, may be regarded as a field of distribution of different chemicals. The pattern of that distribution may be regarded as representative of the distribution of the several constituents and metabolic products and by-products of cells that are growing normally and of cells that are under the influence of penicillin. Therefore, the addition of suitable reagents and careful observation of the reactions that occur in different parts of the test plates might be expected to impart information concerning the mechanism of the action of penicillin on the test organisms. The patterns that develop may be regarded as the result of the interaction of biological "forces," represented by the growth of the test organisms, and of physical "forces," represented by the diffusion of penicillin. The most apparent manifestation of this interaction is the development of zones of inhibition that are readily seen without further treatment on standard 16-hour plates, or that may be revealed easily by proper development with appropriate reagents on plates seeded with organisms and subjected to the diffusion of penicillin for periods as short as 3 hours. Similar chemical evidence may be obtained on standard 16-hour plates, and on 3-hour plates, although the results are sometimes obscured on the former because of the virtually complete destruction and lysis of the cells of the test organism.

The first series of experimental data furnished evidence for a threshold effect involving sulfhydryl groups and, correlatively, dienol groups at the boundaries of the inhibition zones. Inhibition zones on standard 16-hour plates, and on properly developed 3-hour plates, are surrounded by a ring of maximum positive reaction for —SH groups or for dienol groups. Such an intense reaction for —SH groups may be taken as indicative of either an active synthesis of proteins or an active denaturation of proteins that results in the unmasking of bound —SH groups. In other words, an intense positive reaction for —SH groups reveals the site of intense activity of proteinases that may operate in the building up of nucleoprotein complexes or in the denaturation of such complexes.



The rings surrounding the zones of inhibition are also sites of strong positive responses to Millon's reagent, probably indicating the presence of tyrosine, correlative to richness in proteins. The rings of enhanced growth are also the sites of strong positive reactions for phenolic compounds, and for nucleic compounds.

Cytochemical studies of cells of *S. aureus* taken from different regions of the assay plates showed that exposure to bacteriostatic concentrations of penicillin not only tends to prevent cell division, but also effects changes in the location and distribution within the cells of "vacuolar material" responsible for the absorption of vital dyes and, by extension, presumably of other solutes as well. This hypothesis is in accord with the published data of other authors and our own unpublished observations on the localization of reduced silver in normal and in inhibited cells of *S. aureus* following immersion in solutions of silver nitrate, exposure to light, and subsequent development. Vital staining demonstrated that the location of the vacuolar material in cells under the bacteriostatic influence of penicillin corresponds with the site of positive reactions for phenolic compounds (as shown by the reduction of osmic acid or silver nitrate and the adsorption of dyes, such as safranin or basic fuchsin) and with that which stains with basic dyes (such as malachite green and methyl green) that are known normally to stain nucleic acids.

Vendrey and Lipardy (1946) describe the bacterial cell as loaded with ribonucleic acid but surmise that it is located mostly in the cytoplasm which adsorbs basic dyes strongly. The most striking change that we have observed in cells of *S. aureus* affected by a bacteriostatic concentration of penicillin is sharp localization of the absorption or adsorption of basic dyes to the vacuolar material, and sharp restriction of the gram-positive staining reaction to that vacuolar material. This observation may be of fundamental significance, since, as was pointed out above, Gale and Taylor (1946) showed that cells of *S. aureus* under the influence of bacteriostatic concentrations of penicillin may be considered as starving for glutamine, and since it was shown by Stearn and Stearn (1930) that "starving bacteria *gradually* lose gram-positivity passing through a stage where they present a stippled appearance with gram-positive granules throughout." In our experiments with *S. aureus*, we have observed that, under the influence of inhibiting concentrations of penicillin, the gram-positiveness of the cells fades out as the vacuolar material loses its other characteristic properties, namely, positive reaction for dieneols and the ability to retain solutes.

A completely satisfactory physiological interpretation of the mechanism of the action of penicillin on susceptible organisms, however, cannot be based solely on these simple observations involving evidence for the loss of the gram-positive reaction. Any attempt to correlate sensitiveness to penicillin with staining reaction must meet the objection that the gram-negative neisserias are penicillin-sensitive. It should be recalled, however, that using a modification of the gram method, Verhoeff (1940) found he was "able to stain meningococci, in spite of the fact that they are gram-negative," and that Meyrick and Harrison (1942) developed a counterstain for use in the gram technique, whereby "the

gonococcus stains a much deeper color than any other gram-negative organism of this type usually present in smears." A final and ultimate explanation must await a comparison of chemical distribution patterns obtained with various test organisms on assay plates and a general survey of the reactions of gram-negative organisms to penicillin, and especially a thorough investigation of the potentiation of penicillin action toward gram-negatives through the concomitant effect of methionine and threonine, such as has been reported by Schwartzman (1944, 1945, 1946).

In the work that has been reported in this paper and its two predecessors, color reactions obtained in various regions of assay plates and in different parts of the bacterial cells have been interpreted in terms of physiological activity. The possible interference of physical phenomena such as adsorption effects and surface effects or reactions with constituents of the agar has not been overlooked, however (Dufrenoy and Pratt, 1947). It is recognized that the over-all results from experimental data of the type we have presented express the interaction of physiological phenomena with physical phenomena which can be studied separately in terms of surface effects, differential adsorption, metachromatic staining, etc. Although such phenomena have not been discussed in this work, they have been given sufficient consideration and examination in our laboratory to show that they do not prevent the recognition of the physiological events reported above.

#### SUMMARY

Cytochemical and histochemical techniques have been applied to penicillin assay plates according to methods described in previous reports.

Cells of *Staphylococcus aureus* under the influence of bacteriostatic concentrations of penicillin gradually lose their positive reaction to Gram's stain.

The loss of gram-positivity is correlated with changes in the character and distribution of vacuolar material and with the previously reported shift of —SH to S—S at the threshold at the boundaries of the zones of inhibition.

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# SOIL BACTERIA SIMILAR IN MORPHOLOGY TO MYCOBACTERIUM AND CORYNEBACTERIUM<sup>1</sup>

H. J. CONN AND ISABEL DIMMICK

*N. Y. State Agricultural Experiment Station, Geneva, New York*

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When Lehmann and Neumann (1896) first proposed the genera *Corynebacterium* and *Mycobacterium*, the former was intended primarily for the diphtheria organism and the latter for the tubercle and leprosy organisms. In recent years there has been a tendency to broaden both of these genera to include, on the one hand, almost any species showing morphological irregularity and, on the other, various gram-positive nonsporeformers even though showing little or no irregularity in morphology. The original descriptions of these genera were very simple and included only the following essential characters:

*Mycobacterium*. Slender rods with some branching; acid-fast; colonies on agar, dry, wrinkled. Type, *M. tuberculosis*.

*Corynebacterium*. Rods with ends often swollen and club-shaped, banded with alternate streaks of stain, sometimes developing filaments and true branching (by implication non-acid-fast, although this characteristic is not definitely mentioned by the authors until a later edition of their book); growth on agar, soft and nonadherent. Type, *C. diphtheriae*.

Various other characteristics have been listed by later authors for the genus *Corynebacterium*, the most important of which is the so-called "snapping division" of the cells. As this feature is difficult to observe directly, it is usually inferred from the orientation of the cells as described by Kisskalt and Berend (1918), i.e., a tendency to pile up in heaps, with palisade or V-form arrangement. Stress on this characteristic by later authors has undoubtedly been responsible for some unwarranted broadening of the genus, as orientation of this sort can often be observed and does not necessarily indicate the type of cell division which is supposed to be characteristic of *Corynebacterium*.

As a matter of fact, broadening of the two genera has taken place in several directions until they have come to overlap. Moreover, each genus has had species assigned to it which seem to differ more from other species in the same genus than does the type species of one genus from the type of the other. This broadening has taken place along the following lines:

*Mycobacterium*. (1) The inclusion of all acid-fast forms, whether or not branching occurs. (2) The inclusion of many branching forms (Krassilnikov, 1934) whether or not they are acid-fast.

*Corynebacterium*. (1) The inclusion of a rapidly expanding group of "diphtheroids," i.e., animal parasites which are gram-positive and show the type of orientation described by Kisskalt and Berend; a few of these are anaerobic. (2) The inclusion of certain gram-positive plant pathogens, following the lead of

<sup>1</sup> Journal Paper 708, New York State Agricultural Experiment Station, April 2, 1947.

Jensen (1934). (3) The inclusion (also following Jensen, 1934) of strongly aerobic soil bacteria or extremely irregular morphology, showing coccoid and branched forms as well as rods.

The authors' interest in these genera comes from the fact that one of them (Conn, 1928) described under the name of *Bacterium globiforme* an organism which appears as gram-negative, short rods in 24-hour agar slant cultures but as gram-positive cocci after the cultures are 3 to 4 days old. Cultures of this organism have been sent to Krassilnikov and to Jensen; the former is sure it is a species of *Mycobacterium*, the latter that it is a *Corynebacterium*. The latter opinion has been indorsed by Lochhead, who, with one of his associates (Taylor and Lochhead, 1937; Taylor, 1938; Lochhead, 1940), has become one of the leading students of organisms of this type. Their impression in the matter has been summed up by Lochhead (1940) in the following words: "The characteristic *Bact. globiforme* is now believed by us to represent a special group of the corynebacteria with distinctive cultural and physiological properties."

When *Bacterium globiforme* was first described, its author was not unaware of certain resemblances between this organism and either *Mycobacterium* or *Corynebacterium*; but it appeared different in so many ways from the type of either genus that the resemblances were regarded as probably superficial. It was then named as a species of *Bacterium* because that genus was then regarded by the author as a grouping place for species whose relationships were not definitely understood. Since then, however, the conception of *Bacterium* has changed, and it is now usually defined so as to exclude an organism with morphology like that of the species in question.

It must be remembered that when this species was first described the idea of life cycles involving changes in morphology had not been fully accepted, and it took some courage to describe an organism appearing as a gram-negative rod in one stage and a gram-positive coccus in another. Furthermore, the old ideas of monomorphism were then so persistent that it did not occur to the author to make a sufficiently intensive study of the organism to learn whether other morphological forms occurred in its life cycle.

Work on organisms of this type was dropped in the writers' laboratory for several years. It has recently been resumed with the object of comparing cultures of the *Bacterium globiforme* type with strains from other laboratories that have been named as species of *Corynebacterium* or *Mycobacterium*, with the hope of learning how close the relationship between them may be.

#### EXPERIMENTAL WORK

##### *Mycobacterium* Cultures

No extensive study was made of soil cultures that could be regarded as species of *Mycobacterium*. Four cultures, however, were obtained from Jensen labeled, respectively, *Mycobacterium coeliacum*, *M. convolutum*, *M. rubropertinctum*, and *M. crystallophagum*. No similar organisms were found among the available collection of cultures isolated from local soils on ordinary media without special enrichment technique. An attempt was made to secure such forms by isolating

in media to which paraffin-coated pebbles were added, a technique which is regarded as favoring the development of acid-fast; a few partially acid-fast organisms were found, but so late in the work that no careful study of them has yet been made.

The four cultures obtained from Jensen all showed a slight tendency to branch, although not so much variation in morphology was observed as in the organisms to be discussed in the following pages. Three of them were acid-fast, although *M. crystallophagum* was not. All four were gram-positive. They all grew on Mueller's tellurite agar (Difco dehydrated), with typical blackening. All four grew on agar with ammonium phosphate as a sole source of nitrogen; and none of them showed diastatic action on starch.

The authors do not yet feel their work on this group has been extensive enough to warrant an opinion where in the scheme of bacterial classification these organisms belong. It should be remarked that certain students of the pathogenic acid-fast (e.g., Gordon and Hagan, 1936) regard soil acid-fast as very closely related to the pathogens. Accordingly it seems quite likely that Jensen has been entirely justified in describing such forms as species of *Mycobacterium*. It should be emphasized again, however, that Krassilnikov's "mycobacteria" (whose reaction to the acid-fast stain has never been described) do not seem to belong in the genus, but appear rather to be related to the types described below.

#### *Corynebacterium* Cultures

In order to learn how closely the soil bacteria of the *Bacterium globiforme* group are related to *Corynebacterium*, it seemed desirable to obtain a collection of cultures that have been assigned to that genus. The following cultures, as representing what other workers think should go in the same genus as the diphtheria organism, were obtained: 11 cultures of animal and human parasites of diphtheroid nature obtained from P. R. Edwards of the University of Kentucky, W. A. Hagan of Cornell University at Ithaca, H. E. Morton of the University of Pennsylvania, M. Frobisher of the Johns Hopkins Medical School; three strains of *C. helvolum*<sup>2</sup> and one of *C. tumescens* (both soil organisms) from Jensen; and four plant pathogens that have been put in the genus—*C. flaccum-faciens* and *C. fascians* from W. H. Burkholder of Cornell University at Ithaca, *C. poinsettiae* from M. P. Starr of Brooklyn College, and *C. michiganense* obtained many years ago from Miss Bryan, then in the Department of Agriculture at Washington.

*Animal diphtheroids.* The animal and human diphtheroids showed greatest similarity to the type of the genus, *Corynebacterium diphtheriae*. These organisms are comparatively constant in morphology, appearing generally as

<sup>2</sup> Jensen regards this species as synonymous with Zimmermann's *Bacillus helvolus*, renamed *Corynebacterium helvolum* by Kisskalt and Berend. As there is no evidence that Jensen received any strain of Zimmermann's organism for comparison, it is preferred here to think of Jensen's *C. helvolum* as an emendation of the earlier species which stands only if Zimmermann's original organism can no longer be identified. See description at the end of this article.

rods, which are sometimes slightly wedge-shaped or club-shaped, although this morphological peculiarity is not ordinarily as pronounced as in the diphtheria organism itself. The palisade or zigzag arrangement of the cells is common, but truly branched cells have not been observed in the present investigation. The organisms are ordinarily gram-positive; or if gram-variable, the tendency is for the young cells to be positive, the older ones negative. In physiology, the most striking feature is inability to grow on any synthetic medium investigated, a fact which indicates their need of some organic form of nitrogen, or of accessory growth factors, or both. They do not liquefy gelatin or have any visible action on milk, but they are strong producers of acid from sugar.

*Soil organisms.* The soil organisms (Jensen's cultures) proved distinctly different. Their morphological variations are greater, as they often show true branching of the cells, frequently with conidialike spherical bodies on the ends of the branches, and often with the production of larger coccoid bodies (called cystites by Jensen). (See figures 2 and 3.) They are gram-variable, and unlike the animal diphtheroids (figure 4) their tendency is for the older cells to be more strongly positive than the younger. The significance of this behavior to the gram reaction should be more carefully studied in relation to the present-day conceptions of the reaction as dependent on ribonucleic acid; but offhand one would say that there is a significant difference between species which tend to become gram-positive only in older cultures and those which tend to become less strongly so in older culture.

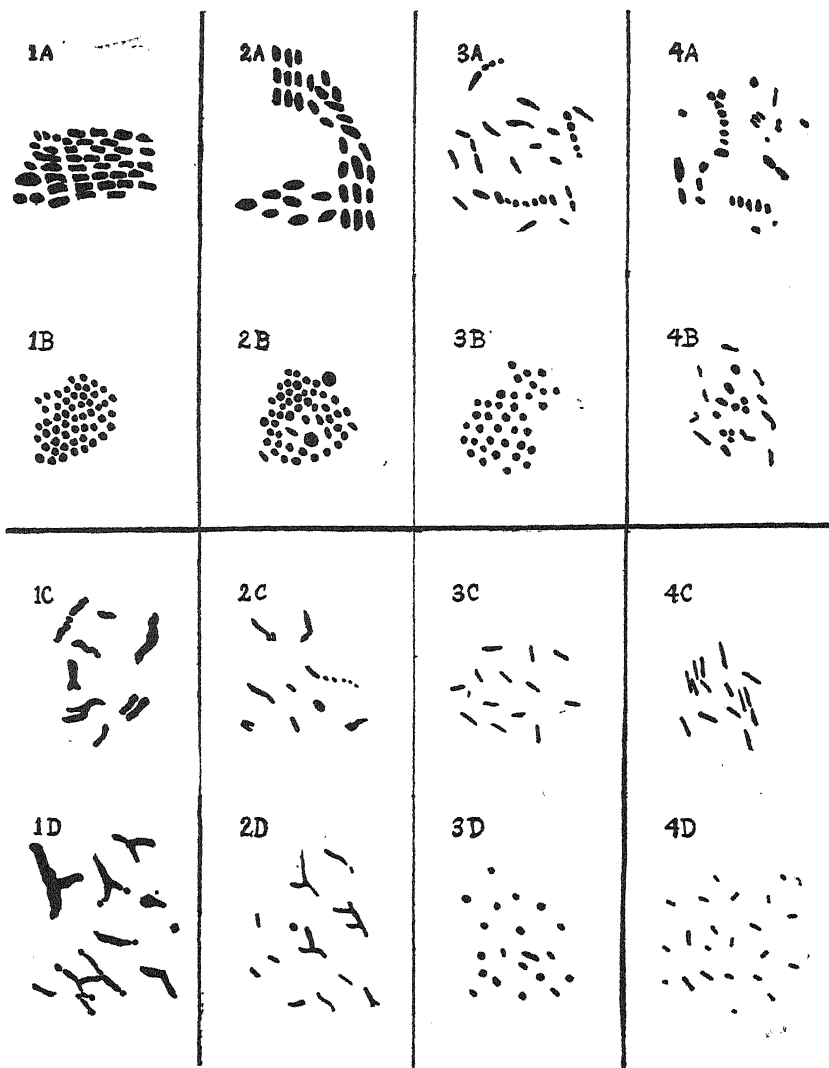
Another striking difference between Jensen's "corynebacteria" and the animal diphtheroids is their ability to grow on synthetic media, with ammonium salts, nitrate, or urea as a sole source of nitrogen, and without the addition of growth accessory factors. They liquefy gelatin and digest milk, but show only weak production of acid from any sugar.

These differences appear to the writers as being of sufficient significance to justify removal of Jensen's species from the genus *Corynebacterium*. Their striking morphology, however, is enough like that of *Mycobacterium* and possibly even of *Nocardia* (Jensen's *Proactinomyces*), that they should be kept close to these genera rather than included in the *Eubacteriales*.<sup>3</sup>

*Plant pathogens.* The plant pathogens present a rather more complicated situation. It has long been recognized that the gram-positive plant pathogens do not fit properly in the genus *Phytomonas*, where several of them have been placed in the past, nor for that matter in any other available genus. Jensen (1934) apparently was the first to place one of them definitely in *Corynebacterium* (i.e., *C. michiganense*). Dowson (1942) transferred two other species (previously *Phytomonas fascians* and *P. flaccumfaciens*) to that genus. This step having already been taken by earlier writers, it was natural for Starr and Pirone (1942)

<sup>3</sup> It should be observed that some recent classifications (e.g., in the forthcoming sixth edition of *Bergey's Manual*) place *Corynebacterium* in the *Eubacteriales*, *Mycobacterium* in the *Actinomycetales*. This arrangement, however, does not agree with the writers' opinion as to the actual relationships of the organisms. See the section of this article on taxonomic considerations.





FIGS. 1-4. SKETCHES TO SHOW PREVAILING MORPHOLOGICAL TYPES ON AGAR AND IN LIQUID MEDIA, IN 1- AND 4-DAY CULTURES

Fig. 1. *Arthrobacter globiforme*.

Fig. 3. *Arthrobacter tumescens*.

Fig. 2. *Arthrobacter helvolum*.

Fig. 4. *Corynebacterium equi*.

The individual sketches are arranged in rows as follows: Row A, 24-hour agar slant culture; Row B, 4-day agar slant culture; Row C, 24-hour culture in sauerkraut glycerophosphate medium; Row D, 4-day culture in sauerkraut-glycerophosphate medium. Preparations shown in rows A and B stained with crystal violet; those in rows C and D with Benian's Congo red method.

on describing a new gram-positive species of a plant parasite (*Phytomonas poinsettiae*) to suggest that it might also be placed in *Corynebacterium*. The

present writers have obtained cultures of all four of these organisms to see whether they are related to any of the other species that have been placed in the genus, and if so to which ones.

Briefly, the writers' conclusions are that all four species (with the possible exception of *Corynebacterium michiganense*) show such differences, both morphologically and physiologically, from the type of that genus, that they clearly belong elsewhere. In fact, they show such differences among themselves that perhaps they do not all belong in the same bacterial genus. Physiologically these plant pathogens seem to stand between the animal diphtheroids and the soil forms mentioned above: they grow on synthetic media, but their proteolytic action is weak or absent. In morphology they differ so much from one another that further discussion is necessary.

*Corynebacterium michiganense* is definitely a nonmotile, gram-positive rod, most nearly like typical diphtheroids of any of the four, although it shows but slight tendency to develop club-shaped forms or other irregularities of morphology. Like the animal diphtheroids, it does not liquefy gelatin, and in fact shows enough similarity to the latter so that Jensen's transfer of this species to *Corynebacterium* may perhaps be justified.

*Corynebacterium fascians* is a nonmotile, gram-variable rod, with a tendency to be more strongly gram-positive in young culture than in old. Although it shows very little morphological variation, it has an appearance on agar slant (dry and yellowish) which strongly suggests relationship to some of the *Actinomycetaceae* (certain *Nocardia* species, for example). As it is slightly proteolytic, it is less like typical *Corynebacteria* forms than the preceding species. Its inclusion in the genus is at least questionable.

*Corynebacterium poinsettiae* and *C. flaccumfaciens* are also yellow chromogens; they are both gram-positive, but are motile with single polar flagella. The latter species often shows sickle-shaped cells with a single, unusually long flagellum at the pole, and one would place the species unquestionably in *Vibrio* if it were not gram-positive. The inclusion of these forms in *Corynebacterium* is highly dubious. The genus is typically one of nonmotile species, and there is little, if any, justification for including types with polar flagella. Such a statement, however, need not reflect on those who proposed placing them in *Corynebacterium*; Jensen, as just shown, has good justification for transferring *Phytomonas michiganensis* to *Corynebacterium*; and Dowson as well as Starr and Pirone could point out close resemblances between that species and the other gram-positive plant pathogens. Nevertheless, *C. poinsettiae* and *C. flaccumfaciens* are distinctly different from the typical *Corynebacterium* species, on the one hand, and from soil forms (as typified by Jensen's cultures), on the other. The present writers hesitate to say just how they should be placed; further study of the question seems indicated.

#### *Cultures from Local Soil*

To compare with the above-mentioned cultures obtained from other laboratories, some 32 strains of organisms like *Bacterium globiforme* in morphology,

isolated from local soils, were studied. Included among them were 14 strains that had been carried in stock for years; the rest were fresh isolations. In comparing these with the cultures from other laboratories it was desired to see whether they belonged in *Mycobacterium* (after Krassilnikov) or in *Corynebacterium* (after Lochhead and Jensen); or if in neither, where they should be placed taxonomically.

A brief study was enough to convince the writers that acceptance of Krassilnikov's conception is out of the question. These cultures can scarcely be called *Mycobacterium*, chiefly because they show no evidence of acid-fastness. Also, they show rather more tendency to branch than typical members of that genus; in this they somewhat resemble *Nocardia* in morphology, but differ from it in having smooth, soft growth on agar (like ordinary bacteria) rather than the dry, wrinkled growth suggestive of the tubercle organism. The fact that Krassilnikov called a culture of *Bacterium globiforme* a species of *Mycobacterium*, whereas Jensen states that the same culture belongs in *Corynebacterium*, is strong evidence that the former's *Mycobacterium* is equivalent to the latter's *Corynebacterium*. Jensen's conception seems more acceptable than Krassilnikov's. As a matter of fact, the similarity of the cultures isolated from local soils to Jensen's *Corynebacterium helvolum* is so great that careful study was needed to show that there really are distinct differences.

*Morphology.* Practically all the cultures selected for this comparative study showed the morphological growth cycle on agar which has been described in the past as characteristic of *Bacterium globiforme*, and is illustrated by the photomicrographs of Conn (1928, p. 6) as well as by sketches 1A and 1B in this paper. Briefly, it may be said that the organisms appear as gram-negative rods in 1-day culture, and as prevalingly gram-positive cocci in older cultures. This particular type of morphology is chiefly characteristic of agar slant cultures. In liquid media the rods tend to elongate and branch, as shown in figures 1C, 1D, and 5. These branching forms are most easily shown by the Benians' negative stain, using Congo red turned blue by treatment with acid; with this technique the apparent diameter of the cells is smaller than when they are positively stained in dry condition, a difference that shows in the sketches of figure 1. Sometimes in liquid cultures about 24 hours old, the nodes of these branched forms appear swollen; and when a gram stain is made of such cells, the swelling proves to be due to a gram-positive coccoid body at the node, the rest of the cell being gram-negative (see figure 5). According to Krassilnikov (personal correspondence) these structures are actually germinating spores. In older liquid cultures similar coccoid bodies seem to be borne like conidia on the ends of the branches, and it can be shown that these are also gram-positive. It is still uncertain whether both of these types of spherical bodies are identical, or whether they are the same as the coccoid forms which show in older cultures on solid media. Krassilnikov's interpretation of the matter, on examination of cultures from this laboratory, is that the organism goes through a regular life cycle: coccoid arthrospores; germinating forms with several branches radiating from the remains of the spore; long rods with a tendency to branch; shorter rods; and finally by a process of

further shortening, the breaking up into coccoid arthrospores. No actual demonstration of such a life cycle has been made here, and clearly no such cycle does occur on agar where only rods and cocci are observed. Moreover, if the forms shown in figure 5 are merely germinating spores, it is difficult to explain how the remains of the spore can retain its gram-positive nature while the rods developing from it are gram-negative. Furthermore, although these forms are observed regularly in the cultures regarded here as typical of what has been called *Bacterium globiforme*, other types apparently closely related show no stages except the rods (more or less elongated and more or less irregular in shape) and the cocci. Another guess, which is probably as justified as that of Krassilnikov's, is that the conidialike bodies formed at the ends of the branches are the same as those seen on old agar slants, whereas those formed at the nodes (which are somewhat larger) are another type of spore similar to what Jensen calls cystites.

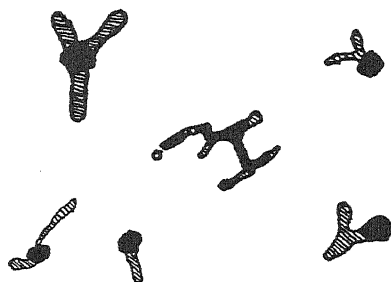


FIG. 5. *ARTHROBACTER GLOBIFORME*, 24-HOUR CULTURE IN SAUERKRAUT GLYCEROPHOSPHATE MEDIUM, STAINED BY THE GRAM METHOD  
Gram-negative structures are shaded; gram-positive structures are solid.

It will be seen from figure 2 that the morphology of Jensen's *Corynebacterium helvolum* is similar. The chief difference is the occurrence of the larger spherical bodies ("cystites") and the persistence of some rod-shaped cells in old agar cultures. Jensen's *C. tumescens* is quite different (figure 3). No other cultures of Jensen's have been available to the authors, but the illustrations in his paper make it evident that *C. helvolum* is the one showing the greatest morphological similarity to what has been recognized here as "*Bacterium globiforme*." Cultures exactly agreeing with Jensen's have not been isolated from local soils.

*Physiology.* When organisms having this type of morphology were first recognized, it was realized that they showed little difference among themselves in physiology; but as nearly all the physiological characteristics were negative ones (except gelatin liquefaction, which was always positive), it was not felt that this apparent similarity was significant. One positive characteristic was the production of small amounts of acid on synthetic media. Jensen lays considerable stress on this; in fact he regards his organisms as distinctly different from *Bacterium globiforme*, because he finds low pH values in carbohydrate media inoculated with *Corynebacterium* strains, whereas *Bacterium globiforme* has been described as producing little acid. It should further be mentioned that it was thought at one time in this laboratory that cultures of this organism could be separated into two species, one producing acid from lactose, the other failing to do so. Subsequent work has shown that none of these differences are of

significance. All of the forms under consideration, regardless of whether they are called *Corynebacterium*, *Bacterium globiforme*, or some other name, can show low pH values after growth on nonbuffered carbohydrate media. It is felt, however, that because of the small amount of actual acid (probably largely  $\text{CO}_2$ ) indicated by the pH changes in the absence of buffer, and because of variations observed in the same cultures on repetition of the tests, such acid production is of no significance and is certainly of no value in the separation of species in the group.

Taylor (1938) divides his cultures (all of which he regards as representatives of *Bacterium globiforme*) into two types: type I utilizes either  $\text{NO}_3$  or urea as a sole source of nitrogen, but type II does not grow on a medium containing either of these nitrogen sources, glucose, and mineral salts. The present writers have observed no such distinction. All of the cultures they have found showing the typical morphology described above grow in media having no nitrogen other than one or the other of the two compounds in question. This either means that no representatives of Taylor's type II have been found locally or that the distinction observed by him has failed to appear under the writers' conditions. It should be remarked that among all the cultures studied here, Jensen's *Corynebacterium tumescens* is nearest like Taylor's type II, but it proves, when in vigorous condition, to be able to utilize either  $\text{NO}_3$  or urea nitrogen.

Another characteristic of the organisms that was at first thought to be of value for classification is the reduction of nitrate to nitrite. Recent investigation, however, indicates that all the organisms of this group do reduce nitrate and that nitrite production can be detected if a synthetic medium of the right consistency is employed (Dimmick, to be published).

At one time in the course of the investigation it was hoped to make use of bacteriophage typing as a means of separating species from one another in this group. This method had, in fact, proved to have value in classifying certain other soil bacteria (Conn, Bottcher, and Randall, 1945). It did not, however, prove adaptable to the group under investigation, either because of lack of specificity in the bacteriophage, or because of easily developed resistance by the bacteria, or both. It was accordingly given up as a criterion for classification.

Recent study has shown one biochemical test which may be constant enough to separate the cultures into two groups—diastatic action on starch. If this characteristic proves constant on further study, a new species must be made for those forms which do not show such action. Also there are some cultures that are yellow chromogens and that may be a distinct species. Because of the extreme variability in physiology shown by these organisms, however, no such species are made at the present time.

#### TAXONOMIC CONSIDERATIONS

As explained above, it is felt that Jensen was mistaken in placing such forms in the genus *Corynebacterium*, because there are striking differences between these organisms and the type species of this genus (the diphtheria organism). Morphologically, however, they show greater similarity to *Mycobacterium* and *Corynebacterium* than to eubacteria. Undoubtedly, therefore, they belong in the

*Mycobacteriaceae*, in spite of certain morphological resemblances to *Nocardia* (*Proactinomyces*). There does not seem to be any genus which exactly fits them in any present system of bacterial classification.

The writers propose for this purpose to revive, by emendation, an old name, *Arthrobacter* Fischer (1895), which as originally proposed was a *nomen nudum*, as no species were named and it was subsequently abandoned even by its author. It is not inappropriate for the present purpose, as it was defined by Fischer as including all nonflagellate, rod-shaped bacteria which produce "arthrospores" as recognized by DeBary. Just what DeBary's arthrospores may have been is not certain, and Fischer later expressed some doubt as to their actual nature; but as the term has been recently revived as a possible name for the conidialike bodies observed in the bacteria now under consideration, an emendation of Fischer's name to apply to them seems permissible.

To discuss the relation of this emended genus to *Mycobacterium* and *Corynebacterium*, certain general points of bacterial classification must be considered. In this the writers prefer to follow the classification given in the fifth edition of *Bergey's Manual*, rather than that which is to be used in the forthcoming sixth edition. This choice is made, first, because the latter classification has, at the time of writing, been distributed only in mimeograph form and, secondly, because in the grouping to be employed in the sixth edition, *Corynebacterium* is placed in the *Eubacteriales* and *Mycobacterium* in the *Actinomycetales*, and the writers prefer to regard these two genera as closely related. According to the fifth edition of *Bergey's Manual*, the differences between these groups may be defined as follows:

- A. Simple and undifferentiated forms, without true branching. Occur as spheres, short or long straight rods, or as curved rods. . . . . *Eubacteriales*
- B. Cells rod-shaped, clubbed or filamentous, with decided tendency to true branching. Conidia may be formed. . . . . *Actinomycetales*
  - I. Rods, or filaments with only slight branching. True conidia not formed. . . . . *Mycobacteriaceae*
  - II. Filamentous forms, often branched, sometimes forming mycelia. Conidia often present. . . . . *Actinomycetaceae*

The family *Mycobacteriaceae*, as described above, may in the writers' opinion be divided into at least the following three genera:

- I. Aerobic slender rods, nonmotile, wholly or partially acid-fast; gram-positive; sometimes clavate or cuneate, or occasionally with rudimentary branching. Many species pathogenic to animals.
 

*Mycobacterium* L. and N.
- II. Aerobic to microaerophilic rods, ordinarily nonmotile, non-acid-fast; gram-positive (most strongly so in young culture); cells often irregularly shaped, clavate, cuneate, or with rudimentary branching, often beaded or barred. Ordinarily require organic nitrogen, growth accessory factors, or both; typically animal parasites, but some dairy forms, possibly some plant pathogens<sup>4</sup>. . . . . *Corynebacterium* L. and N.

<sup>4</sup> If all but the animal parasites ("diphtheroids") are removed from this species, the authors can see no objection to its transfer to the *Eubacteriales*, as proposed for the sixth edition of *Bergey's Manual*.

- III. Strongly aerobic forms, showing rather complicated morphological life cycles, including rods, cocci, clubs, and branched forms; non-acid-fast; gram-variable (young cells usually negative; the older cells, especially those in coccoid form, usually positive); able to live on inorganic nitrogen without added growth accessory substances; typically soil organisms.

*Arthrobacter*, Fischer, emend.

The last-named genus can be characterized as follows:

*Arthrobacter* Fischer, emend.

**Morphology.** Varied, with a tendency to go through a more or less definite life cycle, the most characteristic features of which are gram-negative rods in young cultures and gram-positive coccoid forms (arthrospores?) in old cultures, with intermediate stages that may be clubs, branched forms, or short unbranched filaments. Large (1 to 2  $\mu$ ) spherical bodies are sometimes observed which have been termed "cystites."

**Cultural characteristics.** Growth on surface of solid media soft and smooth, not dry and wrinkled or hard and leathery, as ordinarily in *Mycobacterium* and the *Actinomycetaceae*. Colonies on poured plates ordinarily small (punctiform). Growth in broth usually slow and never profuse.

**Physiology.** Can ordinarily use either ammonium salts or nitrates as sole sources of nitrogen. Can utilize glucose and sometimes other sugars as sources of carbon and energy, but ordinarily without producing sufficient quantities of acid to have appreciable effect on the pH of highly buffered media (e.g., containing peptone). Gelatin usually slowly liquefied. Ordinarily cause blackening of Mueller's tellurite agar.

**Habitat.** Primarily soil.

**Type species.** *A. globiforme* (Conn) Conn and Dimmick.

It seems possible at present to recognize three species:

Species 1. *Arthrobacter globiforme* (Conn) *comb. nov.* (*Bacterium globiforme*, Conn, 1928; *Achromobacter globiforme*, Bergey *et al.*, *Manual*, 3d ed., 1930.) See figure 1, A to D.

Rods in young standard agar culture of fairly regular morphology, 0.6 to 0.8 by 1.0 to 1.5  $\mu$ , becoming (after 2 to 4 days) cocci of about 0.6 to 0.8  $\mu$ ; branching forms with similar cocci and also large spherical bodies (1 to 2  $\mu$ ) in liquid media. Growth vigorous, cream colored (never lemon yellow), on standard agar or on synthetic agar with ammonium salts, nitrate, or urea as the sole source of nitrogen. Diastatic action on starch agar. (Further characterization as given in *Bergey's Manual*.) One of the most abundant organisms in local soil.

(It is possible two other species can be recognized, one differing from the foregoing species in producing lemon yellow on agar, the other in failing to show diastatic action on starch. No names are being assigned to them, however, until the constancy of the differences has been proved.)

Species 2. *Arthrobacter helvolum* (Zimmerman), emend. Jensen; *comb. nov.* (*Bacillus helvolum*, Zimmerman, 1890; *Corynebacterium helvolum*, Zisskalt and Berend, 1918; emend. Jensen, 1934.) See figure 2, A to D.

The three cultures on which this interpretation of the species is based were

secured from Jensen. It is not at all certain that they are the same as Zimmerman's organism. *Corynebacterium helvolum* Kisskalt and Berend, however, was based on a culture received from Zimmerman, and it seems difficult at present to learn just what species it may have been. The present writers, therefore, prefer to regard Jensen's description as an emendation. Based on Jensen's cultures, the species has the following distinctive characteristics:

*Morphology*: Similar to *A. globiforme* in young agar slant culture; older cultures appear as mixtures of rods, small cocci, and the larger spherical bodies, never appearing as though a pure culture of a micrococcus, as is typically the case with the foregoing species; in liquid media, appearance is similar to that of *A. globiforme*. Growth on standard agar; usually lemon yellow, although sometimes merely cream color. Moderately strong to weak diastatic action on starch. This species has not been found in local soil.

Species 3. *Arthrobacter tumescens* (Jensen, 1934) *comb. nov.* (*Corynebacterium tumescens*, Jensen, 1934). See figure 3, A to D.

Morphology on standard agar slant similar to that of *A. globiforme*, but rods in young cultures are more irregular; in liquid media the branching forms are rare or absent. Nonchromogenic. No growth on tellurite agar. No diastatic action on starch. Growth rather scanty on either standard or synthetic media.

This species seems to be something like the type II of "*Bacterium globiforme*" recognized by Taylor (1938), although it apparently utilizes urea and NO<sub>3</sub> nitrogen. It has not been found in local soil. The description is based on a single culture obtained from Jensen.

#### *Possible Other Species*

Jensen places two other species (*Corynebacterium cremoides* and *C. insidiosum*) in the same group with the last two species named, a group which is characterized by great morphological irregularity. He claims the two species to be synonyms of *Bacterium cremoides* Lehmann and Neumann, and of *Aplanobacter insidiosum* McCulloch. The present writers have never received cultures of these forms and do not know whether they should be placed in *Arthrobacter*; according to Jensen's descriptions they seem to be closer to this genus as here defined than they do to true *Corynebacterium*.

#### CONCLUSIONS

There has been a tendency within the last ten or fifteen years to place certain soil bacteria and plant parasites in the genera *Mycobacterium* and *Corynebacterium*; this practice seems to have started independently with Krassilnikov and Jensen in 1934.

The present study has made it evident that Krassilnikov's *Mycobacterium* is the same as Jensen's *Corynebacterium* and is not acid-fast. Partially acid-fast organisms, apparently related to *Mycobacterium*, do occur in soil; but as they do not seem to make up part of the predominant soil flora, they have not been included in the present study.

Special attention has been given to forms found in local soils that are similar to



Jensen's group I of *Corynebacterium* (which show much morphological variation and which he claims are most closely related to the diphtheria organism). It is clear that among these forms should be included *Bacterium globiforme* Conn. It is also evident that they differ so much from *Corynebacterium diphtheriae* that, although probably related to it, they scarcely belong in the same genus. For this group of species the name *Arthrobacter*, emended from A. Fischer, is here proposed, with *Arthrobacter globiforme* (Conn) *comb. nov.* as the type.

A less intensive study has been made of the plant pathogens that have been placed in *Corynebacterium*. It is concluded that *Corynebacterium michiganense* may well belong in that genus, but the inclusion there of *C. fascians* is questionable; *C. flaccumfaciens* and *C. poinsettiae*, however, should not have been placed in it, chiefly because they are motile, with a single flagellum at one pole.

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# THE EFFECT OF IMPURITIES ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN

GLADYS L. HOBBY, TULITA F. LENERT, AND BEVERLY HYMAN

*The Biological Laboratory of Chas. Pfizer & Co., Brooklyn, New York*

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The possible significance of impurities in the chemotherapeutic action of penicillin was first suggested by Cornman (1944) and by Lewis (1944), who observed that impure but not pure penicillin possessed a selective lethal action against rat and mouse sarcoma cells.

Dunham and Rake in 1945 demonstrated that impure penicillin exerted a definite effect on the motility of *Treponema pallidum*, whereas crystalline penicillin had no such activity.

In 1946 Smith showed that germination and root growth is retarded by impure, and not by crystalline, penicillin and stated that the indole-3-acetic acid and phenylacetic acid present in impure penicillins are responsible for this effect.

In a recent communication (Hobby *et al.*, 1946), the comparative efficacy of four forms of purified penicillin and random samples of impure penicillin was described. On a unitage basis, ( $CD_{90}$ ), the relative chemotherapeutic efficacy of penicillins X, dihydro-F, G, F, and K was on the order of 500, 143, 100, slightly less than 100, and 60, respectively. The values for penicillin F were obtained with a preparation which contained impurities, the action of which was not known.

All of the samples of commercial penicillin tested were three to five times more effective than crystalline penicillin G in protecting mice against experimentally produced hemolytic streptococcal infections. The protective action exerted by these impure penicillins was of the same order regardless of the unitage per milligram, or the value of the *Bacillus subtilis*, *Staphylococcus aureus* differential ratio.

In a series of experiments carried out simultaneously with those reported in the present paper, Welch, Randall, and Price (1947) have confirmed the fact that impure preparations of penicillin are more effective than crystalline penicillin G, and in addition have demonstrated that the action of crystalline penicillin G may be enhanced by the addition of penicillin impurities.

## EXPERIMENTAL PROCEDURES AND RESULTS

The present study was undertaken in an attempt to confirm and extend the observations previously reported from this laboratory (Hobby *et al.*, 1946) and to determine, if possible, the factors responsible for the greater activity of impure preparations of penicillin.

*Method.* Throughout this study all comparisons were made by means of mouse protection tests, using the standard procedure previously described (Hobby *et al.*, 1946). Unless otherwise specified, *Streptococcus hemolyticus*, strain

C203Mv, or *Diplococcus pneumoniae*, strain I/230, was used as the infecting organism. One ml of  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  dilutions of a 14-hour rabbits' blood broth culture was injected intraperitoneally into each of a series of 18- to 22-gram white mice. A minimum of 10 mice per dilution of culture was used in each series. Treatment was started exactly  $2\frac{1}{2}$  hours after the infecting dose. The penicillin was administered subcutaneously in 90 per cent peanut oil. Forty per cent of the total dose was administered  $2\frac{1}{2}$  hours, 40 per cent 7 to 8 hours, and 20 per cent 24 hours after infection. Treated animals were observed for a period of 14 days. A control series of untreated animals was included in each day's experiment. The untreated infection was uniformly fatal within 48 hours in the case of *S. hemolyticus*, 96 hours in the case of *D. pneumoniae*.

*Materials used.*<sup>1</sup> Throughout this study crystalline penicillin G having a potency of 1,634 units per mg, by the bioassay method, and a *B. subtilis*, *S. aureus* differential ratio of 1.0 was used. The polariscopic assay of this preparation was 1,635 units per mg. Ultraviolet absorption indicated 100 per cent penicillin G. The crystalline penicillin K used showed a potency of 2,182 units per mg and a differential ratio of 0.36; the crystalline penicillin X, a potency of 1,069 units per mg and a differential ratio of 1.39; the purified penicillin dihydro-F, a potency of 1,675 units per mg and a differential ratio of 0.57.

The impure penicillins used consisted of fractions recovered at various stages in the extraction of penicillin G. The potencies of these varied from 2.8 to 1,028 units per mg; the differential ratio varied from 0.62 to 0.92.

*Action of crystalline and purified penicillin in pneumococcal infections.* The chemotherapeutic effect of highly purified or crystalline penicillins and impure penicillin on hemolytic streptococcal infections in white mice has been reported previously (Hobby *et al.*, 1946). In order to be certain that the difference observed between the actions of impure and purified penicillins on this organism is not characteristic of a single organism only, similar experiments were carried out on a small scale, using *D. pneumoniae*, type I (strain I/230), as the test organism. The results are indicated in table 1.

Considering G as 100, the relative order of efficacy on a unitage basis for penicillins X, dihydro-F, F, G, and K was on the order of 302, 180, 116, 100, and 63, respectively; on a  $CD_{50}$  basis, 204, 138, 100, 100, and 26. The impure preparation tested was more effective against the experimentally produced pneumococcal infection than crystalline penicillin G. The relative efficacy of impure penicillin to crystalline penicillin G, on a  $CD_{50}$  unitage basis, was on the order of 461 to 100; on a  $CD_{50}$  unitage basis, 290 to 100. The relative efficacy of the various penicillins was closely comparable with that previously reported by ourselves

<sup>1</sup> We are indebted to Dr. R. Pasternack and Dr. E. V. Brown of the Department of Research Chemistry of Chas. Pfizer & Co. for the preparation of the crystalline or purified penicillins dihydro-F, G, and K used in this study and to Mr. E. J. Goett of the Department of Research and Development, Chas. Pfizer & Co., for preparation of the impure fractions. We are further indebted to the Lederle Laboratories, Inc., for the preparation of crystalline penicillin X. Analyses of the impure fractions were carried out by Mr. T. Grenfell of the Analytical Department of Chas. Pfizer & Co.

(1946) and by Eagle and Musselman (1946) for *S. hemolyticus* and by Eagle (1947) for *D. pneumoniae*.<sup>2</sup>

*Comparative action of crystalline penicillin G and impure penicillins in hemolytic streptococcal infections.* In view of the fact that impure preparations of penicillin appeared to be more active than crystalline penicillin G, it seemed of interest to determine, if possible, the source and nature of the agent responsible for this phenomenon.

TABLE 1

*Chemotherapeutic action of various forms of penicillin on pneumococcal infections (type I)\* in mice*

FORM OF PENICILLIN .....	X	DIH-F	F	G	K	IMPURE	CONTROLS
Oxford units per mg .....	1,069	1,675	960	1,634	2,182	1,307	
<i>B. subtilis</i> .....	1.39	0.57	0.66	1.00	0.37	0.87	
<i>S. aureus</i> .....							
Total dosage	Therapeutic effect: Percentage of survival						
<i>units</i>							
60	62	30				80	3
100	62	70				82	
150	85		65	62		100	
210	98	80	70	72	45		
300			78	76	42		
400			98	82			
500					60		
600					82		
Relative activities							
CD <sub>50</sub> Biological	302	180	116	100	63	461	
Gravimetric	194	180	107	100	88		
CD <sub>50</sub> Biological	204	138	100	100	26	290	
Gravimetric	131	138	92	100	35		

\* Strain I/230, pneumococcus type I. A minimum of 10 mice was used for each dilution in each set. One ml of a 10<sup>-7</sup> dilution contained 1 to 10 lethal doses of pneumococci; 10<sup>-6</sup>, 10 to 100; 10<sup>-5</sup>, 100 to 1,000; 10<sup>-4</sup>, 1,000 to 10,000.

Crystalline penicillin G and impure penicillins from seven stages in the extraction and recovery of crystalline penicillin G were tested for their relative efficacy in the control of experimentally produced hemolytic streptococcal infections in mice. The total dosages used were 30, 60, 100, 150, 210, and 300 units.

Four of the impure fractions of penicillin tested (preparations 4, 5, 6, and 7)

<sup>2</sup> In a previous communication from this laboratory (Hobby *et al.*, 1946), the relative efficacy against *Streptococcus hemolyticus*, on a gravimetric (CD<sub>50</sub>) basis, was 127, 100, 57, and 40 for penicillins X, G, F, and K, respectively. Eagle and Musselman (1946) reported 260, 100, 50, and 9 for these penicillins, respectively. Recently Eagle (1947) reported values of 160, 100, 83, and 19 against pneumococcus, whereas the data above, on a CD<sub>50</sub> gravimetric basis, indicates a relative efficacy of 131, 100, 92, and 35. The differences undoubtedly indicate variation in technique of injection.

were more effective against this infection than crystalline penicillin G. Eighty-eight to ninety-two per cent protection resulted on administration of 100 units of each of these preparations, whereas 250 to 300 units of crystalline G were required to effect this degree of protection. Impure preparations 1, 2, and 3 were no more effective in controlling this specific infection than crystalline penicillin G. As observed previously (Hobby *et al.*, 1946), there was no correlation between the potency per mg or the differential ratio of the fractions and their degree of protective action (table 2).

TABLE 2

*The chemotherapeutic action of crystalline penicillin G and impure penicillin preparations on hemolytic streptococcal infections in mice*

PENICILLIN	POTENCY	DIFF. RATIO	PHENYLACETIC ACID			PROTECTION* (%) DOSAGE IN UNITS				
			Total	As G	As G: Biopot.	60	100	150	200	300
	u/mg		%	u/mg	u/mg					
Crystalline G	1,634	1.00				46	68	68	82.5	98
Impure										
Prep. 1	1,028	0.89	16	700	66	25	55	65		
Prep. 2	3	0.78	0.5			52	70	75		
Prep. 3	63	0.62	7.5	330	500	65	58	85		
Prep. 4	16	0.70	0.5			45	88	98		
Prep. 5	10	0.76	0.5			52	90	98		
Prep. 6	54	0.92	1.1	3.3	70	77	89	98		
Prep. 7	786	0.75	11.5	490	62	82	92	95		

Throughout this experiment 120 animals were held as untreated controls. Of these 111 (or 92.5%) died within 24 to 48 hours.

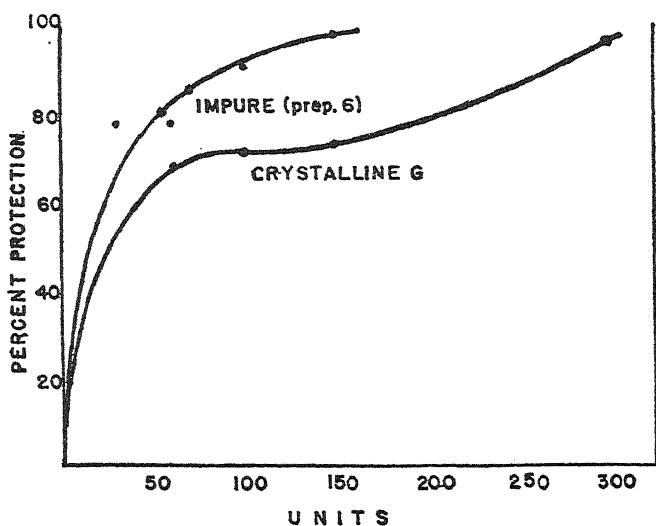
\* Each preparation was tested on a minimum of 40 animals per dosage.

In addition to penicillin and nonpenicillin impurities, each of these fractions contain varying amounts of the degradation products of penicillin. Determinations of the total phenylacetic acid content, the amount of phenylacetic acid as G, and the ratio of phenylacetic acid as G to the biopotency indicated no correlation between these and the efficacy of the fractions *in vivo* (table 2).

It was apparent that the factor responsible for the enhanced activity of penicillin exists at various stages throughout the plant process. It was present in the fermentation liquor and was recovered, with the strongly extractable penicillins and impurities, prior to crystallization of penicillin G. The presence or absence of this factor was not associated with potency, differential ratio, or the phenylacetic acid content.

Impure preparation 6, described above and having a potency of 54 units per mg, was chosen for more detailed study. Comparative studies on the protective action of this preparation and of crystalline penicillin G were made, using a larger series of animals. One hundred units of preparation 6 was again as effective as approximately 250 to 300 units of crystalline penicillin G.

The comparative actions of crystalline penicillin G and impure penicillin preparation 6 are shown in graph 1. Unless otherwise specified, all subsequent experiments were conducted with this crude fraction and the results compared to this curve. Daily checks, using 30, 60, or 100 units of crystalline G, were made to make certain that no change in the effectiveness of crystalline penicillin G had occurred. Some variation in the efficacy of penicillin G in a single strain of animals occurred from day to day. This variation was no greater than occurred in two or more groups of animals of the same strain tested on the same day under the same experimental conditions. The average protection resulting from a given dosage was therefore considered as significant as that on any given day, and unless otherwise specified the average is referred to throughout (graph 1).

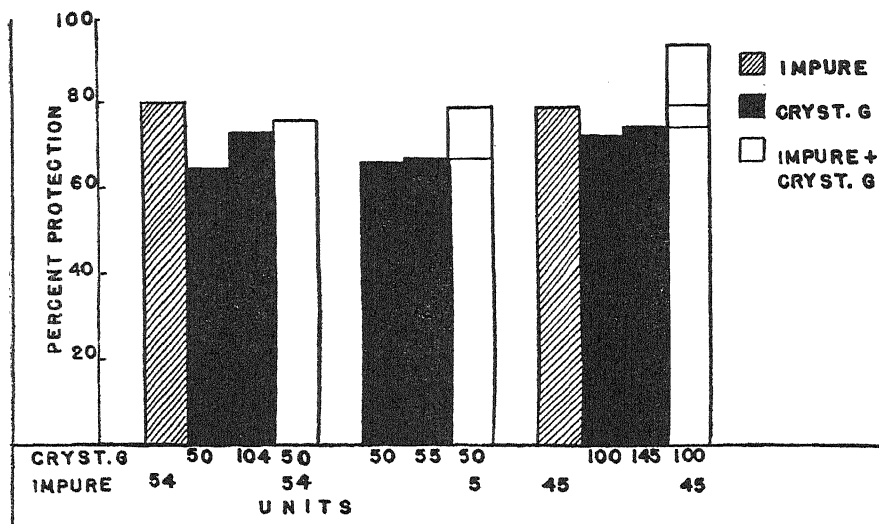


GRAPH 1. COMPARATIVE ACTION OF PURE AND IMPURE PENICILLIN

*Effect of mixtures of crystalline penicillin G and impure penicillin.* In subsequent experiments an attempt was made to enhance the action of crystalline penicillin G by the addition of penicillin impurities. The protective action of an impure preparation of penicillin (preparation 6) in combination with crystalline penicillin G was tested. Mice, infected with hemolytic streptococci (strain C203Mv) in the usual manner, were treated with 100 units of crystalline G in combination with 0.83 mg (45 units) of the impure penicillin. Likewise, mice infected with hemolytic streptococci were treated with 50 units of crystalline penicillin G in combination with 0.1 and 1.0 mg (5.4 and 54 units, respectively) of this preparation of impure penicillin. Throughout this experiment the crystalline penicillin and impure penicillin were dissolved in distilled water and mixed *in vitro* with oil just prior to injection. The results are shown in graph 2.

Although 100 units of this preparation of impure penicillin were sufficient to produce 90 per cent protection (graph 1), the addition of 50 units of crystalline

penicillin G to 1 mg (54 units) of this same preparation of impure penicillin was not sufficient to produce this degree of protection. The protection resulting from the combined action of 1 mg of impure penicillin containing 54 units per mg and 50 units of crystalline G was no greater than that resulting from treatment with 1 mg of this preparation of impure penicillin alone. Administration of 0.1 mg (5.4 units) of impure penicillin in combination with 50 units of crystalline G produced, however, more protection than 50 units of crystalline G alone. Whereas 50 units of crystalline G alone had given only 64 per cent protection, this amount of G in combination with 0.1 mg of impure penicillin (5.4 units) gave 78 per cent protection.



GRAPH 2. THE COMBINED ACTION OF CRYSTALLINE PENICILLIN G AND IMPURE PENICILLIN ON HEMOLYTIC STREPTOCOCCAL INFECTIONS

When 100 units of crystalline G were used, in combination with 0.83 mg of impure penicillin, the resultant protection was again greater than could be obtained from either the amount of crystalline G or the amount of impure penicillin present. Likewise, the percentage of protection was greater than could be obtained with amounts of crystalline G equivalent to the total amount of active penicillin present in the mixture (graph 2).

It was apparent from these experiments, therefore, that the action of crystalline penicillin G could at times be enhanced by the addition of impure penicillin.

*Effect of mixtures of crystalline penicillin G and inactivated impure penicillin.* In subsequent experiments the effect of inactivated preparations of impure penicillin on the activity of crystalline penicillin G was determined. It is recognized that penicillin may be inactivated specifically by penicillinase, which is present in certain preparations of clarase. A concentrated preparation of



penicillinase, made by acetone and alcohol precipitation of clarase,<sup>3</sup> was used in the majority of the experiments. One milligram of this enzyme was sufficient to inhibit completely the action of 2,000 units of penicillin. In order that an excess of penicillinase might always be present, 1 mg was used routinely for inactivation of each 1,000 units of penicillin. For inactivation, penicillinase was added to the solution of penicillin and the mixture incubated at 37 C for 4 hours. At the end of this time samples were withdrawn and tested for potency by the Oxford cup plate method. Only preparations showing, at this stage, no active penicillin were used. The mixtures were then heated at 80 to 85 C for 1 hour to destroy the penicillinase present. Mixtures were stored on dry ice until potency results were available. Samples were again withdrawn for testing, after it had been ascertained that all potency had been destroyed, and 100 units of crystalline penicillin G were added to that amount of inactivated penicillinase mixture, which would contain 0.1 mg of penicillinase. The mixture was incubated for 4 hours at 37 C, diluted, and again tested for potency by the Oxford cup plate method. Only those preparations of inactive penicillin which did not destroy any of the crystalline penicillin G were used.

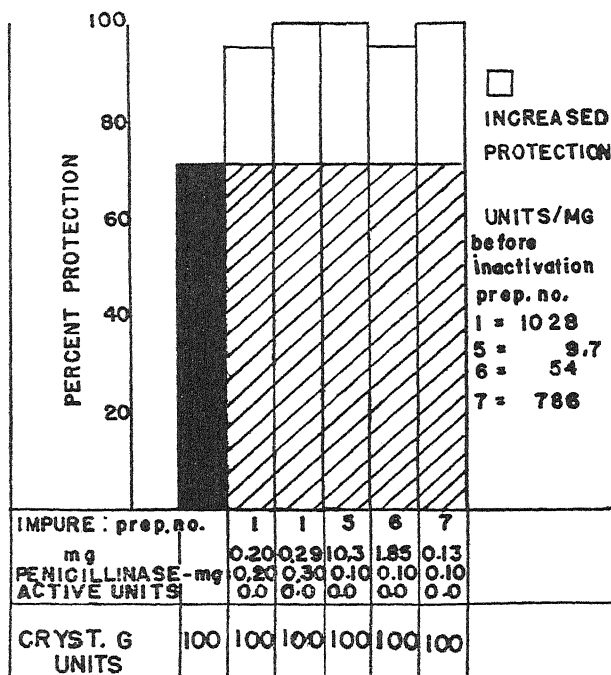
Varying amounts of impure penicillin inactivated by penicillinase in this manner were mixed *in vitro* with crystalline penicillin G. The chemotherapeutic action of these mixtures of crystalline G and inactivated impure penicillin was tested in mice previously infected with hemolytic streptococci, strain C203Mv. Four of the impure preparations previously described (preparations 1, 5, 6, 7; see table 2) were tested. The dosage used consisted of 100 units of crystalline G combined with amounts of inactivated impure penicillin equivalent to that weight which had contained 100 units prior to inactivation. One preparation (no. 6) was also tested in amounts ranging from 0.01 to 1.85 mg (0.54 to 100 units prior to inactivation). Likewise, 25, 50, and 75 units of crystalline G were tested in combination with 1.85 mg of this inactivated impure penicillin (preparation 6). In view of the fact that preliminary experiments had suggested that preparation 1 was no more effective than crystalline penicillin G (table 2), larger amounts of this material, equivalent to 200 and 300 units prior to inactivation, were used.

One hundred units of crystalline penicillin G, in combination with that weight of preparations 5, 6, and 7, equivalent to 100 units prior to inactivation, were sufficient to produce 90 to 100 per cent protection against hemolytic streptococci. Preparation 1, which previously had been shown to be no more effective in the active form than crystalline penicillin G, likewise was capable of enhancing the

<sup>3</sup> A suitable preparation of clarase, obtained from the Takamine Laboratories, was dissolved in water, 7 volumes of acetone were added, and the mixture was allowed to stand in the refrigerator overnight. The brown supernatant fluid was decanted, the precipitate was dissolved in distilled water, and two volumes of 95 per cent alcohol were added. The mixture was again cooled in the refrigerator until precipitation was complete. The mixture was then centrifuged, the supernatant fluid discarded, and the precipitate redissolved in distilled water. Alcoholic precipitation was repeated three times or until a white, flocculent precipitate appeared. The precipitate was dissolved in a small volume of water, frozen, and dried *in vacuo*. By this procedure a readily soluble and highly active preparation of penicillinase was obtained.

protective action of 100 units of crystalline G to 95 to 100 per cent when weights of the inactive material equivalent to 200 or 300 units, prior to inactivation, were used (graph 3).

One hundred units of crystalline penicillin G, in combination with as little as 0.01 mg of preparation 6 inactivated by penicillinase, was sufficient to produce over 90 per cent protection against hemolytic streptococci. As indicated previously, 100 units of crystalline G alone produced only 71 per cent protection (graph 4).



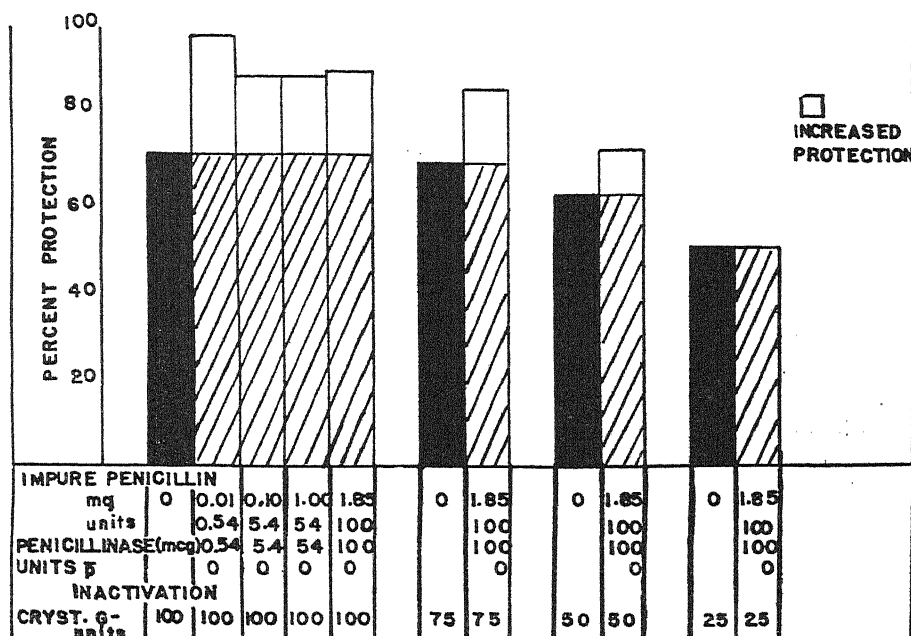
GRAPH 3. EFFECT OF INACTIVATED IMPURE PENICILLIN ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G (100 UNITS)

Seventy-five units of crystalline G in combination with 1.85 mg of inactivated impure penicillin were adequate to produce 86 per cent protection, whereas 75 units of crystalline penicillin G alone would be expected to produce only 62 per cent protection. Fifty units of crystalline G in combination with this amount of inactivated impure penicillin produced 72 per cent protection as compared to 62 per cent with crystalline G alone. On the other hand, 25 units of crystalline G combined with this amount of inactivated impure penicillin produced the same degree of protection as would be expected with crystalline G alone. The degree of protection due to the crystalline penicillin G, therefore, was increased 10 to 27 per cent by the addition of suitable amounts of inactive impure penicillin. Control experiments using 1.24 to 2.68 mg of inactivated impure penicillin alone indicated that this material had no protective action itself (graph 4).

It was apparent that the protective action of crystalline G was enhanced by the addition of inactivated impure penicillin as well as by the addition of active impure penicillin.

*Effect of penicillin degradation products on crystalline penicillin G.* In the inactivation of penicillin by penicillinase, a marked increase in the degradation products present in these impure preparations naturally occurred. Attempts were therefore made to test the effect of these substances on the activity of crystalline penicillin G *in vivo*.

Crystalline penicillin G was inactivated by penicillinase, heated to destroy the enzyme, and tested in the manner previously described for impure penicillin.



GRAPH 4. EFFECT OF INACTIVE IMPURE PENICILLIN ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G

There was no indication either that any active penicillin or that any active penicillinase remained. This inactive form of penicillin was then made up to its original potency by the addition of active crystalline penicillin G and the resultant mixture tested for its chemotherapeutic action against hemolytic streptococci in mice. There was no enhancement of the action of crystalline penicillin G. Indeed, the results suggest that these degradation products may even decrease the action of crystalline G.

In view of the possibility that small amounts of residual active enzyme might have been present in these experiments, further studies were carried out with crystalline penicillin G that had been inactivated by alkali. Again there was no enhancement of the action of crystalline G.

Preliminary experiments with a preparation of crystalline G high in penicillic acid have suggested that this substance not only plays no part in the enhancement of crystalline penicillin G, but may possibly decrease the efficacy of penicillin. Previous data have indicated that phenylacetic acid likewise is probably not correlated with the factor responsible for the enhancement of crystalline penicillin G *in vitro* (table 2).

No attempt has been made to evaluate the effect of each of the degradation products of crystalline penicillin G. Those formed by alkaline or enzymatic inactivation of this form of penicillin, as well as penicillic acid and phenylacetic acid, however, appeared to have no ability to enhance the action of crystalline penicillin G.

*Effect of inactivated penicillinase on crystalline penicillin G.* Since large amounts of enzyme were used in the inactivation of the impure penicillin, the possible effect of this substance on the activity of crystalline penicillin G was of interest. Protection studies were therefore made of mice infected with hemolytic streptococci and subsequently treated with crystalline penicillin G combined with varying amounts of the preparation of penicillinase used above. In each case the enzyme was heated at 80 to 85 C for 1 hour and then tested, before being used, to make certain that no activity remained. Dosages consisting of 100 units of crystalline G combined with 0.00054 to 0.10 mg of inactivated penicillinase prepared from clarase and 100 units of crystalline G combined with 0.54 mg of a preparation of inactivated penicillinase made from *Bacillus* sp.<sup>4</sup> were tested.

As little as 0.054 to 0.10 mg of the inactivated penicillinase prepared from clarase was sufficient to increase the protective action of 100 units of crystalline penicillin G from 71 per cent to 88 to 90 per cent. On the other hand, 0.54 to 1.0 mg of the inactivated enzyme prepared from *Bacillus* sp. enhanced the action of crystalline G to only 78 to 80 per cent. Penicillinase itself, therefore, was probably not entirely, if at all, responsible for the effect. The remarkable effect of small amounts of the preparation of penicillinase obtained from clarase suggested that some other substance must exist in clarase which is responsible for its activity (graph 5).

*Effect of clarase on crystalline penicillin G.* Clarase, lot no. 2404,<sup>5</sup> which contained no penicillinase, was chosen for subsequent study. Again with hemolytic streptococci as the infecting organism, mouse protection studies were made. Infected animals were treated subcutaneously with 100 units of crystalline penicillin G combined with 1.0 mg of clarase. Under these conditions 100 units of crystalline G was adequate to produce 97 per cent protection. One milligram of clarase gave no protection.

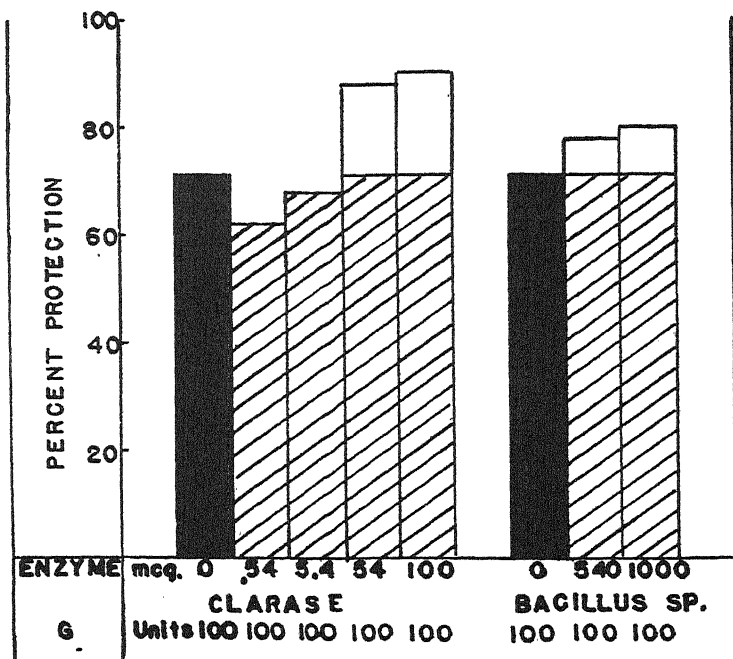
It is apparent therefore that whereas the impure preparations of penicillin are more effective than crystalline penicillin G, and whereas the impurities from

<sup>4</sup> We are indebted to Dr. A. J. Liebmman of Schenley Corporation for the culture of *Bacillus* sp. (no. 569) used. Penicillinase was prepared by acetone and alcohol precipitation of broth cultures of this organism. One milligram of the preparation used was sufficient to inactivate 100 units of penicillin.

<sup>5</sup> Clarase, lot no. 2404, containing no penicillinase was obtained through the courtesy of Mr. W. A. McIntyre and Dr. Mildred Adams, Takamine Laboratories, Clifton, New Jersey.

penicillin can enhance the activity of crystalline G, the activity of penicillin can also be enhanced by other substances not normally present in impure penicillin. Concentrates of penicillinase prepared from clarase will produce such an effect. That this effect is probably not due entirely, if at all, to the penicillinase itself is implied by the fact that a preparation of clarase containing no penicillinase can similarly enhance the action of crystalline G, whereas a preparation of penicillinase from another source has little effect.

*Effect of inactivated impure penicillin on the chemotherapeutic action of penicillins X, dihydro-F, and K.* In view of the fact that the action of crystalline penicillin



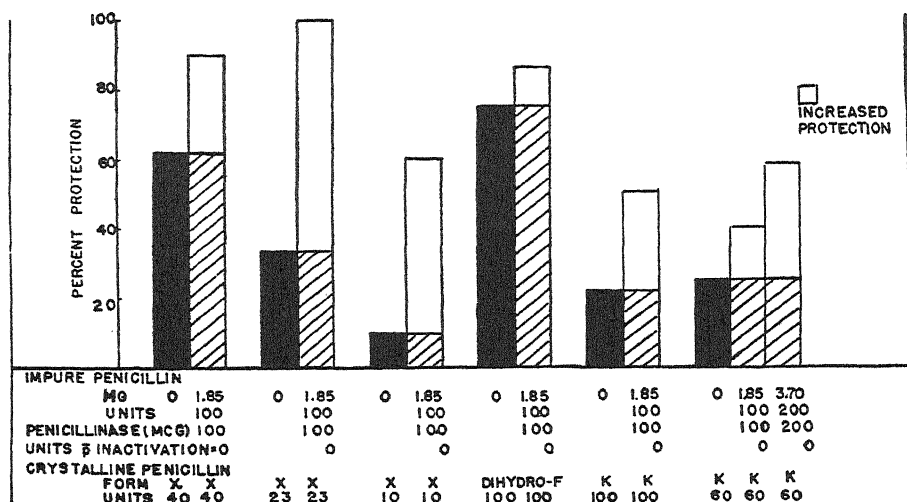
GRAPH 5. EFFECT OF INACTIVATED PENICILLINASE ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G

G could be enhanced by inactivated crude penicillin preparations, and in view of the fact that impure preparations of penicillin are also more effective than the other forms of penicillin, it seemed of interest to determine whether or not one could demonstrate a similar enhancement of the activity of other penicillins by impurities.

Impure penicillin, preparation 6, inactivated in the manner previously described, was again used. An amount (1.85 mg) which had contained 100 units of penicillin prior to inactivation was mixed with 100 units of highly purified penicillin dihydro-F, with 10, 23, and 40 units of crystalline penicillin X, and with 100 units of crystalline penicillin K. In addition, 3.70 mg of this inactivated preparation were also mixed with 100 units of crystalline penicillin K. These mixtures

were tested for their therapeutic action against hemolytic streptococcal infections in the usual manner.

Under these conditions, 100 units of penicillin dihydro-F was adequate to protect 86 per cent of mice against infection. This preparation of dihydro-F, alone, in a dosage of 100 units, afforded only 75 per cent protection. Ten, twenty-three, and forty units of crystalline penicillin X in the presence of inactive impure penicillin gave 60, 100, and 90 per cent protection, respectively. In the absence of impurities, one would obtain only 10, 34, and 62 per cent protection, respectively, from this preparation of crystalline penicillin X. With 100 units of crystalline penicillin K, in the presence of an amount of inactivated impure penicillin which had contained 100 units prior to inactivation, only 50 per cent protection resulted. In the absence of impurities, however, this preparation of



GRAPH 6. EFFECT OF INACTIVE IMPURE PENICILLIN PREPARATIONS ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLINS X, DIHYDRO-F, AND K

penicillin K, in a dosage of 100 units, gave only about 22 per cent protection. Approximately 230 units would have been necessary to produce 50 per cent protection. The addition of a larger amount of inactivated impure penicillin to this preparation of crystalline penicillin K did not further enhance its effectiveness.

In a subsequent series, 1.85 mg of inactive impure penicillin (preparation 6) combined with 60 units of crystalline K produced 40 per cent protection, whereas 3.70 mg combined with this amount of crystalline K gave 57.5 per cent protection. Sixty units of K alone in the strain of mice used for this particular experiment were capable of protecting only 25 per cent of the animals infected<sup>6</sup> (graph 6).

<sup>6</sup> This set of experiments was conducted with a different strain of mice from that used for previous experiments. The degree of protection due to 60 units of crystalline penicillin K in this strain was similar to that due to 100 units in the strain of mice previously used.

It is apparent therefore that under suitable conditions, the action of all of the penicillins (X, dihydro-F, G, and K) may be enhanced by the presence of impurities. The effect on penicillin K, however, is less regular.

*Action of mixtures of purified or crystalline penicillins.* In view of the fact that many preparations of impure penicillin contain, in addition to impurities, a mixture of the various penicillins, further studies were carried out to determine the effect of these penicillins on each other.

In preliminary experiments, a preparation of mixed penicillins recovered from impure material was used. This preparation (120) having a potency of 1,197 units per mg and a *B. subtilis*, *S. aureus* differential ratio of 0.95 was separated into two fractions: (1) a fraction containing a mixture of penicillins and having a potency of 1,574 units per mg and a *B. subtilis*, *S. aureus* differential ratio of 0.97 and (2) a fraction containing predominantly impurities and having a potency of only 190 units per mg and a differential ratio of 1.03. All three fractions were tested for their chemotherapeutic activity. In a dosage of 100 units, the original preparation and the fraction containing only 190 units per mg each protected 85 per cent of the animals infected, whereas the purified preparation of mixed penicillins afforded only 46 per cent protection.

Although high in penicillin G, this purified preparation contained small amounts of penicillins F, dihydro-F, and possibly K. Analyses indicated 91 per cent penicillin G—the remaining 9 per cent probably consisting predominantly of dihydro-F, with not more than 2 to 3 per cent K. The amount of penicillin X present was negligible. The amounts of these penicillins present were incapable of enhancing the action of the penicillin G.

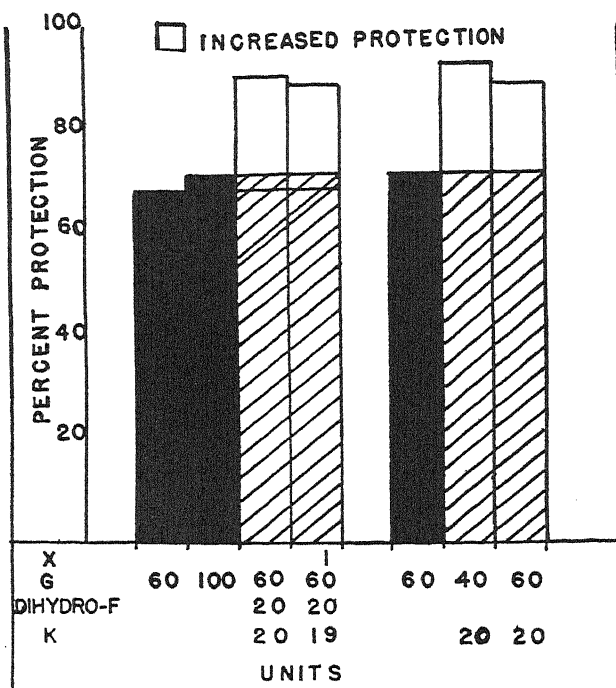
In subsequent preliminary experiments, mixtures of known amounts of highly purified or crystalline penicillins were tested. Sixty units of crystalline G were mixed (1) with 20 units of dihydro-F, 19 units of K, and 1 unit of X, and (2) with 20 units of dihydro-F and 20 units of K. The chemotherapeutic action of these mixtures was greater than would have been expected from 100 units of dihydro-F, G, or K. Indeed, it was as high as would be expected from 60 to 100 units of impure penicillin or of crystalline penicillin X. No greater protection was obtained with the mixture containing 1 per cent penicillin X, however, than with that completely devoid of X (graph 7).

The preliminary experiments described indicated that whereas a mixture of penicillins, containing dihydro-F, F, and G with a small amount of K and probably no X, had no greater chemotherapeutic action against hemolytic streptococcal infections than crystalline G, a mixture containing, in addition to crystalline G, 20 per cent dihydro-F and 20 per cent K was far more potent than crystalline G alone. Whether the latter effect was due to the penicillin K alone, to the larger amount of dihydro-F in the latter mixtures, or to the combined action of dihydro-F and K could not be determined from these data.

The combined action of crystalline penicillins G and K were therefore tested in a limited series of animals. Twenty units of crystalline penicillin K were mixed (1) with 40 units of crystalline G and (2) with 60 units of crystalline G. Sixty units of crystalline penicillin G were used as control.

Sixty units of crystalline G afforded 72 per cent protection,<sup>7</sup> whereas the combination of crystalline penicillin G and K afforded 88 to 92 per cent protection. It was apparent that crystalline penicillin K can enhance the activity of crystalline penicillin G and that the effect is greater than would be anticipated from the total number of units present.

*The effect of host susceptibility on penicillin dosage.* In the course of the experiments described above, an opportunity to observe the effect of alteration in host susceptibility on the penicillin dosage required to protect against hemolytic streptococcal infections in mice presented itself.



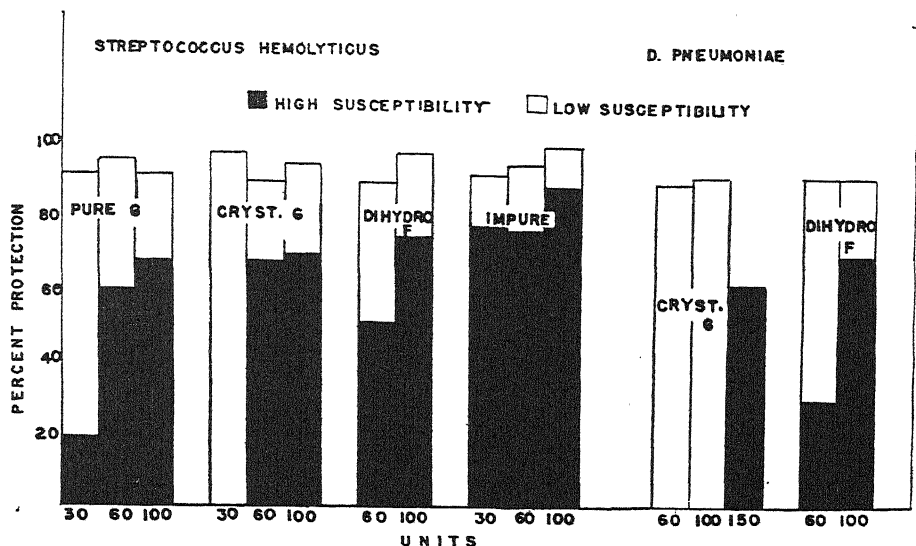
GRAPH 7. CHEMOTHERAPEUTIC ACTION OF MIXTURES OF PENICILLIN

A spontaneous infection due to an organism believed to be similar to *Corynebacterium murium*, described by Condrea (1930), developed throughout the mouse colony. This infection was benign in nature and at no time fatal to the spontaneously infected mice. Shortly after the infection was first observed, however, it became apparent that this strain of mice was no longer as susceptible to hemolytic streptococcal and pneumococcal infections as in the past. Although the infections were still fatal to this strain of animals, death occurred slowly and, in the higher dilutions, less uniformly. Coincident with this shift in host suscepti-

<sup>7</sup> Owing to the fact that a strain of mice differing from that employed in the majority of previous experiments was used here, the percentage of protection due to 60 units of crystalline G differed slightly from that previously described.



bility, the amount of penicillin necessary to protect against such streptococcal and pneumococcal infections decreased markedly. Whereas 250 to 300 units of penicillin G had previously been necessary to give approximately 90 per cent protection against hemolytic streptococcal infection, 15 to 30 units of this same material were now adequate to produce this degree of protection. Likewise 60 units of penicillin dihydro-F were now as effective as 210 units had previously been, and 30 units of a preparation of impure penicillin were as effective as 100 to 150 units in the past. Similarly the amount necessary to protect against pneumococcal infections in this strain of mice was lowered (graph 8).



GRAPH 8. COMPARISON OF THE CHEMOTHERAPEUTIC ACTION OF PENICILLINS IN MICE OF HIGH AND LOW SUSCEPTIBILITY

It was not possible during this time to demonstrate agglutinins or precipitins against hemolytic streptococci in the sera of these mice. The serum did, however, possess a marked antibacterial action against this group of organisms. Zones of inhibition varying from 11 to 35 mm in size were observed with samples of undiluted sera when tested against *Staphylococcus aureus* (H) by the Oxford cup plate method used for penicillin assays. Normal sera showed no zones of inhibition. The heating of samples of whole blood to 80 to 85 C for 2 hours, during which time coagulation of the red blood cells occurred, was sufficient to prevent diffusion of the active principle, although spot plate tests indicated that it was still present in an active form. The substance responsible for the antibacterial action was stable at 56 to 60 C for 2 hours, as indicated by the fact that the size of the zones of inhibition of *Staphylococcus aureus* was not decreased.

*Streptococcus hemolyticus*, strain C203Mv, was inhibited in broth by one sample of pooled sera from these mice in dilutions up to 1:512, whereas pneumococci were inhibited by dilutions up to 1:1,024 and staphylococci by dilutions up to 1:128.

As the infection in the colony regressed, newly bred animals of the same strain gradually regained their original susceptibility to pneumococcal and streptococcal infections. Simultaneously, the amount of penicillin necessary to induce protection approached the original levels.

In view of the fact that a shift in susceptibility sufficient to alter the course of infection without producing a complete immunity could cause such a marked change in the effective chemotherapeutic dosage, it seemed of interest to compare several known strains of mice. Four standard pure-line strains were used. Although the difference in susceptibility was again evidenced only by the time necessary to produce death, a marked difference existed in the amount of penicillin necessary to effect protection.

#### DISCUSSION

Impure penicillin is a more effective chemotherapeutic agent than crystalline penicillin G against at least four microorganisms belonging to different species. It is probable that such a difference may be demonstrable against a wide variety of organisms.

Dunham and Rake (1945), working with *Treponema pallidum*, first suggested that impure penicillin may possess greater chemotherapeutic activity than crystalline penicillin G.

The differences in the action of the pure penicillins in contrast to preparations of impure penicillin was first discussed in detail in a recent communication from this laboratory (Hobby *et al.*, 1946). Impure penicillin was shown to be 3 to 5 times more effective against hemolytic streptococcal infections in mice than crystalline penicillin G. Indeed, such impure penicillin was more effective than any of the five forms of purified penicillin with the exception of penicillin X.

In the present report the same difference in activity has been demonstrated against pneumococcal infections in mice. Welch, Randall, and Price (1947) likewise have demonstrated recently a similar effect against infections due to *Eberthella typhosa*, and Rake, Dunham, and Donovick (1947) have confirmed their original observations on *Treponema pallidum* and have extended them to include the action of impure and pure penicillins on pox viruses grown in the chick embryo.

The substance responsible for the greater activity of the impure penicillins in the streptococcal and pneumococcal infections is present in the original fermentation liquors and may be recovered, during the purification of penicillin, in those fractions in which penicillin G is recovered. It does not crystallize with penicillin G, however. It is relatively heat-stable. Preliminary studies suggest that it is readily dialyzable. Only small amounts are necessary to enhance the activity of crystalline penicillin G. As indicated by the data of Welch *et al.*, and also by ours, it is effective on penicillins X, dihydro-F, and K as well as on penicillin G.

It has been shown previously (Hobby *et al.*, 1946) that there is no correlation between the presence of this factor and the source of the penicillin, its

potency in terms of units per mg, or its composition as evidenced by the *Bacillus subtilis*, *Staphylococcus aureus* differential ratio. These facts have been amply confirmed by Welch and his associates (1947).

That the more common degradation products probably are not in themselves responsible for enhancing the activity of crystalline penicillin G is suggested by the fact that enzyme- or alkali-inactivated crystalline G has failed to enhance active crystalline penicillin G when mixed with it. Likewise, no correlation exists between the presence, in impure penicillins, of the substance which enhances chemotherapeutic activity and the amount of phenylacetic acid present. A preparation of crystalline G, high in penicillinic acid, has shown no greater chemotherapeutic efficiency than crystalline penicillin G itself.

That the factor which enhances the action of crystalline penicillin G in streptococcal and pneumococcal infections is not specifically correlated with the penicillin impurities only is suggested by the data presented in this communication. Certain apparently dissimilar agents may produce this effect. The penicillinase used for inactivation of impure penicillin, as well as the substances normally present in impure penicillin, have the ability to increase the efficacy of crystalline penicillin G. The penicillinase used in these studies was prepared from clarase. That the effect is not due to the penicillinase itself is indicated by the fact that a preparation of clarase, containing no penicillinase, was also highly effective, whereas a preparation of penicillinase from another source possessed little or no activity. Preliminary experiments indicate, furthermore, that certain mixtures of penicillins are more effective than crystalline G alone and that crystalline penicillin K may enhance the activity of crystalline G.

Whether these enhancing substances act directly on the crystalline penicillin or whether they produce their effect through an alteration of some mechanism within the body is not known. That the latter is true, at least in part, is suggested by preliminary experiments in which inactivated impure penicillin was administered simultaneously with crystalline penicillin G but at different sites within the body.

Tompsett, Schultz, and McDermott (1947) have recently demonstrated differences in the ability of the various penicillins to be bound by the albumin component of serum. Whereas only about 50 per cent of penicillin G is bound, as much as 90 per cent of penicillin K may be bound. Clowes and Keltch (1946) have demonstrated, furthermore, that larger amounts of penicillin K than G are removed from solution when exposed in a Warburg apparatus to the action of various amounts of muscle or liver slices. The effect of penicillin impurities or of penicillin K on the binding action of crystalline penicillin G is not known.

The possible existence of an antibacterial agent effective *in vivo*, but not *in vitro*, must be considered. Esters of penicillin showing such activity were described by Meyer, Hobby, and Dawson (1943). More recently Ramon, Richou, and Ramon (1946) have described a substance present in crude penicillins that possesses "antidotal" properties, and Miller and Boor (1947) have described protective action against certain bacterial endotoxins. Since the organisms used throughout this study elaborate a number of toxins, it is con-

ceivable that a substance capable of neutralizing one or more of these might enhance the protective action of crystalline penicillin.

The data presented herein are preliminary in nature. Neither the nature of the enhancing substance present in impure penicillin nor the mechanism by which it acts has been determined. That one or more such substances do exist seems undoubtedly true. That a variety of dissimilar substances, including ones not specific for impure penicillins, may similarly enhance the action of crystalline penicillin G in streptococcal and pneumococcal infections is suggested. It seems possible that the factor (or factors) responsible for this form of enhancement may differ in nature or in their mechanism of action from those responsible for the enhancement of the action of penicillin on sarcoma cells, vaccinia virus, or bacterial toxins.

The individual experiments described represent, in many instances, only small numbers of animals and, therefore, are probably not biostatistically valid. The total study, however, represents over 25,000 animals and indicates a definite trend.

Differences in host susceptibility may alter the amount of penicillin necessary to protect against certain infections. The relative efficiency of the various penicillins is therefore more significant than the actual dosage necessary to protect. An exact comparison of the chemotherapeutic value of various agents in animals is probably possible only in pure-line strains of relatively constant susceptibility.

#### CONCLUSIONS

As previously reported, impure penicillin is 3 to 5 times more effective than crystalline penicillin G in protecting animals against experimentally produced hemolytic streptococcal infections in mice. The presence of this factor is not correlated with the potency in units per mg or with the *Bacillus subtilis*, *Staphylococcus aureus* differential ratio. Likewise it is not correlated with the amount of phenylacetic acid present. There is no evidence that it is associated with the degradation products of penicillin.

As in the case of hemolytic streptococcal infections, impure penicillin is more effective than crystalline penicillin G in protecting animals against experimentally produced pneumococcal infections in mice. On a unitage basis ( $CD_{50}$ ) the relative chemotherapeutic efficacy of penicillins X, dihydro-F, F, G, and K against this infection, under the experimental conditions used, was on the order of 302, 180, 116, 100, and 63, respectively; on a gravimetric basis, 194, 180, 107, 100, and 88, respectively.

The factor in impure penicillin which enhances the chemotherapeutic activity of crystalline penicillin G is present in the original penicillin fermentation liquor and may be demonstrated during the extraction of penicillin in those fractions in which penicillin G is recovered.

The chemotherapeutic activity of crystalline penicillin G may be enhanced by dissimilar substances not specific for impure penicillin. Furthermore, crystalline penicillin K may enhance the activity of crystalline penicillin G. The

effect of penicillin G plus K is greater than would be anticipated from the total number of units present.

The chemotherapeutic activity of penicillins X, dihydro-F, and at times K may also be enhanced by penicillin impurities.

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# BACTERIAL DISPERSION BY SONIC ENERGY

R. F. SHROPSHIRE

*Research Division, Raytheon Manufacturing Company, Waltham, Massachusetts*

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When a suspension of dispersed bacteria is subjected to intense sonic energy, sufficient to cause cellular rupture, there is a progressive decrease in the turbidity of the liquid with continued time of exposure. This change in turbidity can be used as a method of following the cellular disruption in that, when the proper wave length of light is employed, there is a direct relationship between the concentration of organisms and the absorption coefficient, or the transmittancy. This relationship is valid if the organisms are well dispersed. When they exist in aggregates, the extent of the reduction of the light passing through the liquid in which they are suspended will be somewhat less than if the same number of individuals were uniformly dispersed throughout the suspending medium.

In connection with some exploratory work, which was being done on the effect of sonic energy on various bacteria, in which a turbidimetric method of evaluating bacterioclasis was utilized, it was observed that, when the organisms under exposure existed in aggregates, the first detectable optical change was an *increase* in turbidity. Microscopically, it was confirmed that this increase was correlated with the extent to which aggregates were being separated.

If this dispersion effect could be utilized, it might well prove of value in connection with experimental procedures, such as nutrition studies, which are based on an assumption that such colonies which subsequently develop have been derived from single individuals.

It was for the purpose of preparing viable suspensions of well-dispersed organisms which normally exist in aggregates, such as clusters or chains, that the experiments to be described were undertaken.

## METHODS AND EQUIPMENT

A laboratory model of a device, made by the Raytheon Manufacturing Company of Waltham, Massachusetts, for the application of intense sound energy to small quantities of liquids was utilized. It consists of a stainless steel cup, the bottom of which is a diaphragm that is vibrated by a laminated nickel structure connected to it. This magnetostrictor transducer is driven by an electronic power oscillator having an output of approximately 60 watts, at a frequency of about 9,000 cycles per second.

Absorption measurements were made by means of a balanced cell type of photoelectric colorimeter the light source of which had been corrected by means of filters to have its principal transmission at approximately 6,500 angstroms. The absorption coefficients which are plotted as ordinates on the accompanying

graphs represent the difference between the natural logarithm of the instrumental scale readings when no organisms are present in the suspending liquid and the natural logarithms of the instrumental scale readings under various experimental conditions when organisms are present.

The conditions in which organisms existed, whether singly or in aggregates, was also determined microscopically.

#### EXPERIMENTAL RESULTS

A small quantity, usually 20 ml, of a 24-hour culture of the organisms under study was placed in the cup of the transducer, and subjected to intense sonic energy for varying lengths of time. Prior to treatment the absorption coeffi-

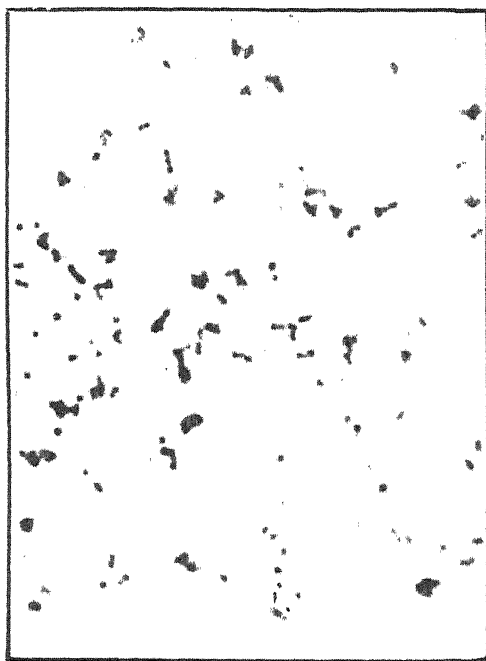


FIG. 1. UNTREATED STAPHYLOCOCCUS ALBUS CULTURE

cient of the suspension was determined turbidimetrically, against sterile culture medium as a blank. At suitable intervals during the course of the exposure to sound, turbidity measurements were made. Thus the change in turbidity with time of treatment could be plotted, and an optimum time for best dispersion empirically established. It usually coincided with that point of the curve corresponding to maximum turbidity. As stated before, the ordinates on the accompanying graphs represent differences between the natural logarithms of scale readings and are not absolute values. They are a function of the scale of a particular instrument, and the dimension and shape of the cells in which the liquids were held while turbidities were measured. However, the relationships



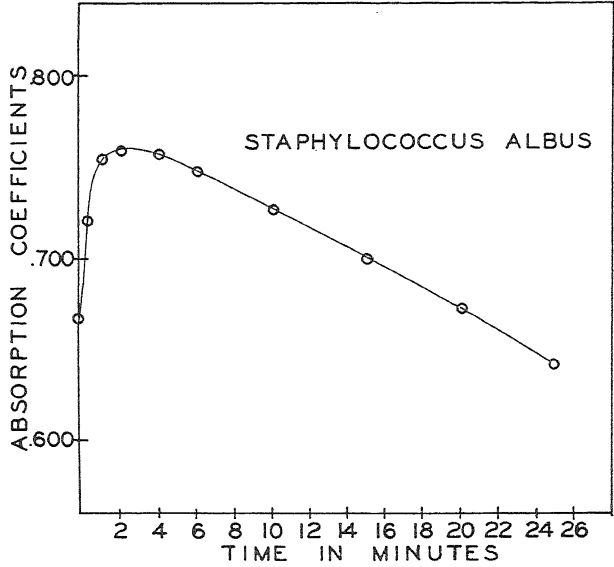


FIG. 2. TURBIDITY CHANGES IN STAPHYLOCOCCUS SUSPENSION UNDER SONIC ENERGY



FIG. 3. DISPERSED STAPHYLOCOCCUS ALBUS AFTER SONIC EXPOSURE

are relative and similar measurements made on any instrument will give comparable curves.

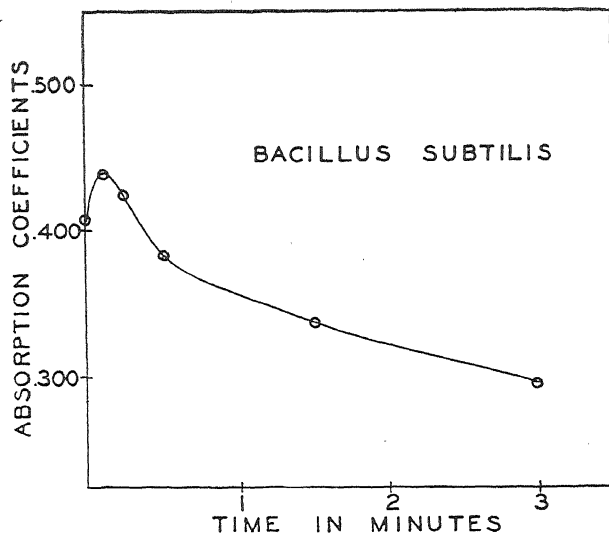


FIG. 4. TURBIDITY CHANGES IN *BACILLUS SUBTILIS* SUSPENSION UNDER SONIC ENERGY

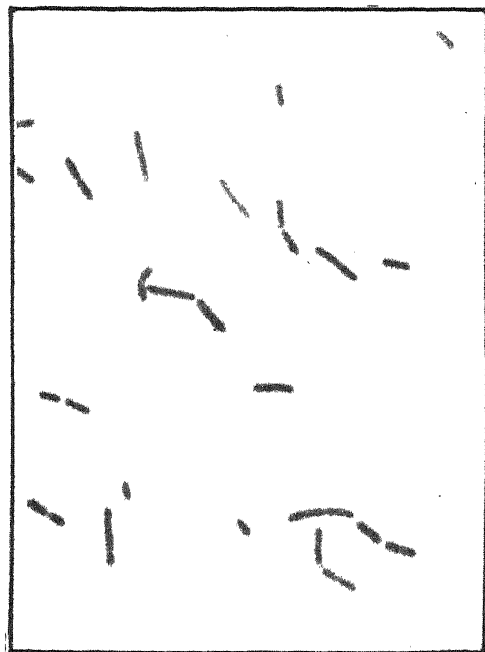


FIG. 5. *BACILLUS SUBTILIS* SUSPENSION BEFORE TREATMENT

In order to avoid what would be rather pointless repetition, only three dispersions of organisms will be reported in detail. Out of many cultures which

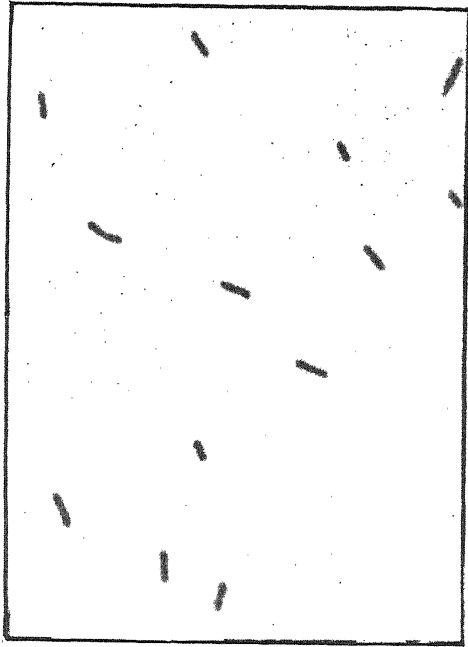


FIG. 6. *BACILLUS SUBTILIS* DISPERSION AFTER SONIC EXPOSURE

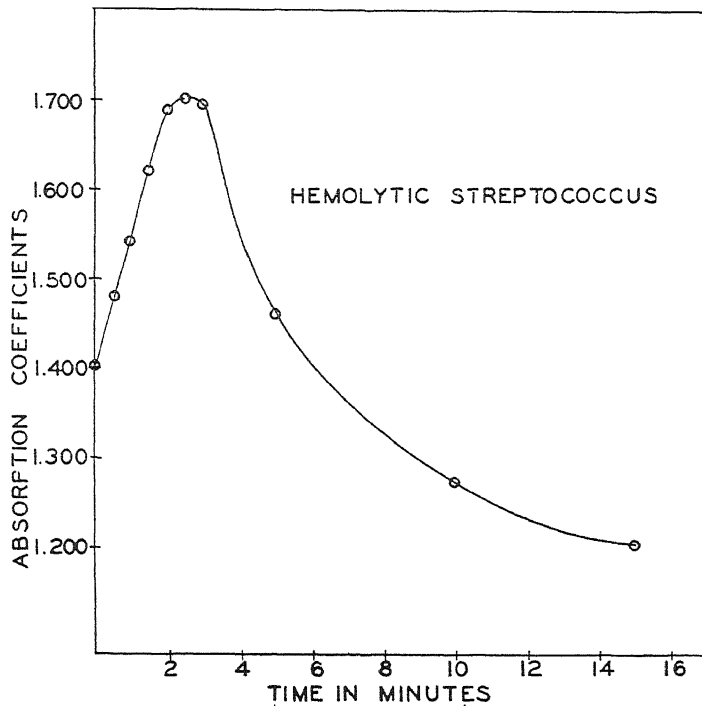


FIG. 7. TURBIDITY CHANGES IN *HEMOLYTIC STREPTOCOCCUS* UNDER SONIC ENERGY

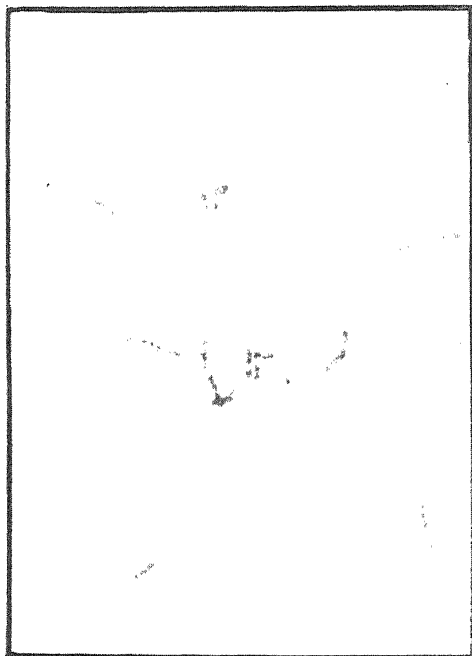


FIG. 8. STREPTOCOCCUS SUSPENSION BEFORE TREATMENT

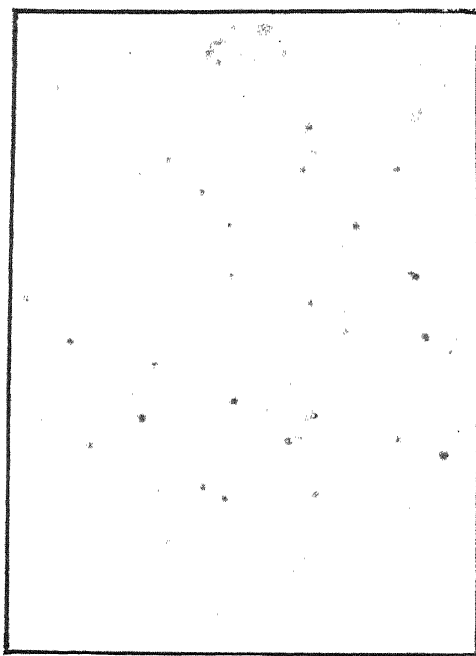


FIG. 9. STREPTOCOCCUS DISPERSION AFTER SONIC EXPOSURE

were studied these three were selected as characteristic of types of aggregates which have been dispersed.

*Staphylococcus albus*. A 24-hour culture of *Staphylococcus albus* was grown in nutrient broth and its turbidity determined. A slide prepared at this time was photographed and is shown in figure 1. As can be seen, there were numerous aggregates. A 20-ml portion of the culture was placed in the cup of the transducer and exposed to intense sound energy at a frequency of almost 9,000 cycles per second. At selected intervals of time the turbidity of the liquid under treatment was determined. These turbidities expressed as absorption coefficients are shown in figure 2. It will be noted that there is a definite increase in turbidity up to 3 minutes, after which it decreases. This decrease in turbidity represents cell rupture, and is, in the case of aggregated organisms, an effect which follows their dispersion.

The microscopic appearance of the same culture shown in figure 1 but after 20 minutes' exposure to sonic energy is shown in figure 3. It will be noted that the individual organisms are well separated.

*Bacillus subtilis*. A 24-hour culture of *Bacillus subtilis*, grown in nutrient broth, was treated under similar conditions to the *Staphylococcus albus* described in the foregoing experiment. Absorption coefficients obtained turbidimetrically are shown in figure 4. Figure 5 shows the appearance of the organisms before treatment, and figure 6 shows their appearance after 3 minutes of sonic exposure.

*Hemolytic streptococcus, group B*. A 24-hour culture of *Hemolytic streptococcus, group B*, grown in nutrient broth was exposed to sonic energy, and was followed both turbidimetrically and microscopically. Figure 7 shows the change in turbidity with continued time of treatment; figure 8 shows the culture as it appeared before treatment; and figure 9 is its appearance after 10 minutes of sonic exposure.

#### SUMMARY AND CONCLUSIONS

Turbidimetric changes in three cultures exposed to sonic energy have been described and the dispersion of aggregates illustrated.

A method is suggested for the preparation of well-dispersed suspensions of viable organisms which normally have a tendency to form aggregates.

The exposure of cultures to sonic energy for suitable selected lengths of time provides a means for the separation of bacterial aggregates and the preparation of dispersed individual organisms.



# A MOUSE PROTECTION METHOD FOR THE ESTIMATION OF ANTIGENIC PNEUMOCOCCAL POLYSACCHARIDE IN SOLUTION

CURTIS SANDAGE<sup>1</sup> AND ORTON K. STARK

*Miami University, Oxford, Ohio*

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In a study involving the preparation and testing of pneumococcus capsular polysaccharide, the need for a rapid method of assay of preparations of unknown antigenic potency became evident. Most bacterial vaccines are standardized on the basis of cell counts or total nitrogen determinations. Since such vaccines usually contain somatic protein as the principal antigen, determination of the number of cells or of the quantity of nitrogen is often a reliable criterion of antigenicity. Obviously, neither procedure can be used for the standardization of capsular carbohydrate preparations.

Heidelberger and Kendall (1932) developed a method for the quantitative estimation of specific carbohydrate in solution which is based on the precipitation of this material by homologous type-specific antiserum. By this method, they were able to determine as little as 0.01 mg of type III pneumococcus polysaccharide. The procedure is quite satisfactory for many purposes, but it does not necessarily measure the antigenicity of the substance under test. For example, Felton (1934) found that heating type I pneumococcus polysaccharide in acid solution destroyed from 50 to 87 per cent of its immunizing activity, although the precipitin titer was not altered. Avery and Goebel (1933), working with type I pneumococcal capsular polysaccharide (subsequently referred to as SI), found that the deacetylated product precipitated specific antibody from homologous antiserum but that it was not antigenic when tested in mice. It is evident in this case that the method based on the precipitin reaction is not a reliable index of antigenicity. The method of Heidelberger and Kendall is applicable only when the specific carbohydrate is obtainable in a state of purity. Even then, a given weight of the pure material may vary widely in antigenicity owing to the effect of different methods of purification.

Sevag (1934) reported that mice treated with 0.0001 mg of SI survived 1,000 fatal doses of type I pneumococcus. Schiemann *et al.* (1931), using highly purified SI, found that the minimum amount necessary to produce demonstrable active immunity when injected into white mice was 0.01  $\mu$ g. Since mice are so responsive immunologically to antigenic SI, it seemed that a mouse protection method might offer an extremely sensitive means of evaluating the antigenicity of experimental preparations.

## EXPERIMENTAL

A sample of highly purified SI was obtained<sup>2</sup> and tested in white mice. Animals weighing 18 to 22 grams were injected intraperitoneally on 4 successive

<sup>1</sup> The Wm. S. Merrell Co., Fellow.

<sup>2</sup> Dr. Michael Heidelberger, College of Physicians and Surgeons, Columbia University, kindly supplied this material.

days using a daily dose of 0.25 ml of tenfold dilutions of the SI in saline. Five days after the last immunizing dose, the mice were challenged by intraperitoneal injections using a type I pneumococcus culture. The virulence of the test culture was such that 1.0 ml of a  $10^{-9}$  dilution was regularly fatal to control animals within 60 hours. Dilutions of  $10^{-7}$ ,  $10^{-8}$ , and  $10^{-9}$  were used in order to be certain that some animals would receive 100 or more MLD. In this work, there has been no need to use the LD<sub>50</sub> method.

The data shown in table 1 indicate that the minimum quantity of SI affording complete protection is 0.01  $\mu$ g. Using this material as a standard of potency, various crude and partially purified preparations were assayed by determining the highest dilution of the unknown which gives corresponding protection against type I pneumococcus.

The results of a representative test are shown in table 2. The material under test was a partially purified SI solution obtained from a broth culture. It is evident that 1:5,000,000 is the highest dilution of the unknown affording mouse

TABLE 1

*Determination of the minimal quantity of standard SI which provides immunity against D. pneumoniae, type I*

Number of mice surviving 72 hours after inoculation.

IMMUNIZING DOSE OF STANDARD SI	DILUTION OF CHALLENGING CULTURE			
	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$
None (control)		0/6	0/6	6/6
1.0 $\mu$ g	6/6	6/6	6/6	
0.1 $\mu$ g	6/6	6/6	6/6	
0.01 $\mu$ g	6/6	6/6	6/6	
0.001 $\mu$ g	0/6	0/6	2/6	

protection in this case. Since this dilution contains a quantity of SI equal in antigenic activity to 0.01  $\mu$ g of the standard, the quantity of active SI in the original solution can be calculated as 50 mg per ml.

Both tables 1 and 2 illustrate the definite end point which has been obtained routinely by this method. In table 1 it can be seen that whereas quantities of the standard SI from 1.0  $\mu$ g to 0.01  $\mu$ g are completely effective in protecting mice, 0.001  $\mu$ g is ineffective. The lowest concentration of SI affording complete protection is considered to be the end point. No significance is attached to the average survival time for individual groups, even though mice receiving less than 0.01  $\mu$ g occasionally appear to survive somewhat longer than control animals. The necessity for statistical treatment of data is thus eliminated. In order to obtain these definite end points, it is necessary to standardize the conditions of culture and the virulence of the organism used for challenging. Mice of the proper weight from four sources have been used for these tests with completely consistent results.

The results obtained in an attempt to apply this method for the estimation of



SI in body fluids are shown in table 3. A rabbit weighing 3 kilograms was injected intravenously using 25 mg of SI. One hour later the rabbit was bled

TABLE 2

*Determination of the minimal dose of unknown SI solution which provides immunity against D. pneumoniae, type I*

Number of mice surviving 72 hours after inoculation.

	DILUTION OF CHALLENGING CULTURE			
	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>
Controls				
Saline		0/6	0/6	6/6
Standard SI, 0.01 µg	6/6	6/6	6/6	
Dilutions of unknown SI				
1:500,000	6/6	6/6	6/6	
1:5,000,000	5/6	6/6	6/6	
1:50,000,000	0/6	0/6	1/6	

TABLE 3

*Estimation of SI in rabbit serum by determining active immunity developed in mice in response to injection of the serum*

Number of mice surviving 72 hours after inoculation.

	DILUTION OF CHALLENGING CULTURE			
	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>
Controls				
Saline		0/6	0/6	6/6
Normal serum 1:10	0/6	0/6	1/6	
SI standard, 0.05* µg	6/6	6/6	6/6	
Dilution of serum† from rabbit injected with 25 mg SI				
1:10	6/6	6/6	6/6	
1:1,000	6/6	6/6	6/6	
1:10,000	4/4	4/4	4/4	
1:100,000	0/4	0/4	0/4	

\* Diluent was normal serum 1:10.

† Serum from blood obtained by cardiac puncture 1 hour following intravenous injection of SI.

from the heart. Basing the calculations on body weight, the serum obtained should have contained approximately 200 µg per ml. Calculations based on the results given in table 3 indicate that it contained more than 100 µg but less than 1,000 µg per ml. No attempt was made to determine the quantity more accu-

rately, though it could doubtless be done by using additional dilutions of the unknown. It appears that the presence of body fluids does not interfere with the test, although naturally occurring immune substances in serum must be considered.

#### DISCUSSION

The usual mouse protection tests, both active and passive, emphasize the determination of the number of lethal doses which treated animals resist rather than the determination of the quantity of antigen required to produce significant immunity. The passive mouse protection test of immune serum is, nevertheless, an indirect means of determining the quantity of antibody present (Heidelberger, Sia, and Kendall, 1930). The principle involved in active mouse protection tests is quite different in that the response of the mouse to the antigenic stimulus determines the amount of antibody formed. It would appear that the determination of the minimal quantity of an antigen which elicits active immunity may be more significant as a measure of antigenicity than the number of lethal doses of the test organism which the mouse will resist. The method presented accomplishes this objective and gives easily interpreted end points.

In preliminary work it appears that the time necessary to complete such a test can be shortened considerably. In one experiment it was found that a single injection of 1.0 ml of the material under test produced the same results as did 4 consecutive daily injections of 0.25 ml. This would shorten the time required for a determination by 3 days. It may not be necessary to allow 5 days between immunization and challenging of mice. In another experiment comparable results were obtained after a 4-day waiting period. Since significant deaths occur within 72 hours, this schedule permits a test to be completed within 8 days.

The method described has been limited in application to pneumococcal materials which are antigenic in mice and for which an acceptable comparison standard can be obtained. There is no reason to believe that it could not be applied to the evaluation of other antigenic materials of a similar nature. The choice of dosage schedule, waiting period, and the standard to be used is arbitrary and can be planned to suit individual needs.

#### SUMMARY

A mouse protection method for the estimation of antigenic pneumococcal polysaccharide in solution has been described.

The principle of the test is based on the immune response of white mice to minute quantities of antigenically active polysaccharide.

The procedure should be a useful supplement to methods based on the precipitin reaction because of its sensitivity and technical simplicity. Furthermore, the method described does not require standardization of antisera or purification of the antigen under test.

This procedure provides a measure of antigenic potency rather than a measure of precipitable polysaccharide.

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# SEROLOGICAL STUDIES OF THE GENUS *XANTHOMONAS*

## III. THE *XANTHOMONAS VASCULARUM* AND *XANTHOMONAS* PHASEOLI GROUPS; THE INTERMEDIATE POSITION OF *XANTHOMONAS CAMPESTRIS*

R. P. ELROD AND ARMIN C. BRAUN

*Department of Animal and Plant Pathology, The Rockefeller Institute for Medical  
Research, Princeton, New Jersey*

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Previous studies (Elrod and Braun, 1947a) indicated that the genus *Xanthomonas* showed evidence of antigenic divisibility. Since the individual species of the genus cannot easily be differentiated by the physiological determinative procedures ordinarily used in bacteriology, it was suggested that serological information might be utilized as an aid in the classification of these organisms. Further study by means of agglutinin absorption in one of the divisions, arbitrarily designated as the *X. translucens* group (Elrod and Braun, 1947b), argued against the possibility that all described species within this group represented distinct serological entities. Nevertheless, it was possible to define certain specific immunological components within this group which apparently were correlated with host specificity. Discrepancies in this respect were indicated by the serological identity of organisms isolated from such widely separated sources as pumpkin, begonia, and certain grasses.

The present paper deals with a continuation of serological study of three other immunological divisions of the genus *Xanthomonas*—the *X. vascularum*, *X. phaseoli*, and *X. campestris* groups. It was previously observed (Elrod and Braun, 1947a) that, by using heavily mucoid cultures, a relationship could be shown to exist between the “*vascularum*” and “*phaseoli*” groups. The *X. vascularum* type organism agglutinated in the *X. phaseoli* group antiserums, but the reciprocal reaction was not observed. It was found that, by freeing the cultures of the mucoid material, this unilateral relationship was lost; there were no agglutination reactions between the two groups. The two organisms in the *X. campestris* group, *X. campestris* and *X. barbareae*, tended to link the two groups in question. All of the cultures in the *X. vascularum* and *X. phaseoli* groups agglutinated in *X. campestris* and *X. barbareae* antiserums. The latter two organisms reacted in all of the individual antiserums of the two larger groups. In the present investigation the nature of this intermediate role was investigated, and the members of the “*vascularum*” and “*phaseoli*” groups were compared by means of agglutinin absorption.

### ORGANISMS OF THE *XANTHOMONAS VASCULARUM*, *XANTHOMONAS* PHASEOLI, AND *XANTHOMONAS CAMPESTRIS* GROUPS

The species and subspecies that compose these three groups have a wide and varied host range. Table 1 presents information pertaining to natural hosts,

isolates employed in the study, and the original authority for the species in question. It is to be noted that the susceptible hosts extend through many genera and families of the higher plants. *Xanthomonas vascularum* is apparently specific for sugar cane, whereas *X. vesicatoria* has a known host range limited to certain of the *Solanaceae*, more particularly pepper and tomato. *X. vesicatoria* v. *raphani* is infective for pepper and tomato as well as for the radish. In this

TABLE 1  
*Species and subspecies with representative hosts in the Xanthomonas vascularum, Xanthomonas phaseoli, and Xanthomonas campestris groups*

SPECIES	ISOLATES STUDIED	NATURAL HOST OR HOSTS	AUTHORITY
<i>X. vascularum</i>	XVI, XV46	Sugar cane ( <i>Saccharum officinarum</i> )	Cobb, 1893
<i>X. vesicatoria</i>	XV3, XV4, XV7, XV8, XV13, XV14	Tomato ( <i>Lycopersicon</i> spp.) Pepper ( <i>Capsicum</i> spp.)	Doidge, 1921
<i>X. vesicatoria</i> v. <i>raphani</i>	XV16, XV16A	Radish ( <i>Raphanus</i> spp.) Tomato ( <i>Lycopersicon</i> spp.) Pepper ( <i>Capsicum</i> spp.)	White, 1930
<i>X. papavericola</i>	XP5, XP17	Poppy ( <i>Papaver rhoeas</i> )	Bryan and McWhorter, 1930
<i>X. hederæ</i>	XH1, XH6	Ivy ( <i>Hedera helix</i> )	Arnaud, 1920
<i>X. incanae</i>	X13	Stock ( <i>Malhiola incana</i> )	Kendrick and Baker, 1942
<i>X. taraxaci</i>	XT11	Russian dandelion ( <i>Taraxacum kok-saghyz</i> )	Niederhauser, 1943
<i>X. campestris</i>	XC2, XC3, XC10, XC15	Cabbage, cauliflower ( <i>Cruciferae</i> spp.)	Pammel, 1895
<i>X. campestris</i> v. <i>armoraciae</i>	XC4	Horse radish ( <i>Armoraciae</i> spp.)	McCulloch, 1929
<i>X. barbareae</i>	XB1, XB2	Winter cress ( <i>Barbareae vulgaris</i> )	Burkholder, 1941
<i>X. phaseoli</i>	XP1, XP14, XP28	Beans ( <i>Phaseolus</i> spp., <i>Dolichos</i> spp., <i>Lupinus</i> spp.)	Smith, 1897
<i>X. phaseoli</i> v. <i>fuscans</i>	XP18, XP19, XP26	Beans ( <i>Phaseolus</i> spp.)	Burkholder, 1930
<i>X. geranii</i>	XG1, XG1a, XG3, XG4	Geranium ( <i>Geranium</i> spp.)	Burkholder, 1937
<i>X. pelargonii</i>	XP7, XP8, XP15	House geranium ( <i>Pelargonium</i> spp.)	Brown, 1923
<i>X. malvacearum</i>	XM2, XM13, XM14	Cotton ( <i>Gossypium</i> spp.)	Smith, 1901

regard it is similar to *X. barbareae*, *X. campestris*, and *X. campestris* v. *armoraciae* in being pathogenic on some of the *Cruciferae*. The poppy is host to *X. papavericola*; *X. hederæ* infects ivy; *X. incanae*, stocks; *X. taraxaci*, the Russian dandelion; and *X. barbareae*, winter cress. *X. geranii* and *X. pelargonii* attack geraniums in different genera, *Geranium* and *Pelargonium*. Cotton is affected in world-wide distribution by *X. malvacearum*. Beans of different genera are susceptible to *X. phaseoli* and *X. phaseoli* v. *fuscans*. The latter owes its distinction to the elaboration of a brown pigment.

## EXPERIMENTAL RESULTS

Without recourse to repetition the reader is referred to table 1 and table 2, part A (Elrod and Braun, 1947a) for the cross-agglutination aspects of the three serological groups under consideration.

Random sampling of absorbing combinations was not successful in producing specific serums in the *Xanthomonas vascularum* group. As a consequence, reciprocal absorptions were conducted between all eight organisms, with a few exceptions concerning *X. incanae*. In no case did these mirror absorptions indicate that any two of these species were serologically identical. Nor was it possible to produce a specific antiserum for the homologous organisms by absorption with any one of the heterologous types. The patterns produced by these absorptions were varied, giving the impression that we were dealing with a multitude of group-specific factors. It was felt that the use of different organisms in absorbing combinations would produce specific serums. This laborious procedure was not deemed warranted. In table 2 are indicated typical results obtained when individual serums were absorbed with single cultures. On absorbing *X. vesicatoria* (XV14) antiserum with *X. vesicatoria* v. *raphani* (XV16), all heterologous agglutinins except those for *X. hederæ* (XH1) were removed. When the foregoing antiserum was absorbed by the latter organism, the agglutinins for the absorbing culture and *X. vascularum* (XV1) were obliterated. When *X. papavericola* (XP5) was used to absorb this serum, all agglutinins remained except those for XP5 and *X. incanae* (XI3). The probable antigenic uniformity of *X. vesicatoria* is indicated by the complete removal of antibody by XV7 or XV13, other isolates of this species. Dissimilar patterns are produced also by absorbing *X. vascularum* (XV1) antiserum. Generally, reactive components remained for most of the antigens concerned when this serum was acted upon by *X. hederæ* (XH1), *X. papavericola* (XP5), *X. vesicatoria* v. *raphani* (XV16), or *X. campestris* v. *armoraciae* (XC4). The latter organism was found to have closer immunological affinities for the *X. vascularum* group than for *X. campestris*. Absorption of the antiserum for *X. campestris* v. *armoraciae* (XC4) by *X. vascularum*, *X. vesicatoria*, and *X. papavericola* left sizable group components. Absorption, however, with *X. vesicatoria* v. *raphani* (XV16) removed all agglutinins, homologous and heterologous. Reciprocally, a large homologous-reacting fraction remained when the *raphani* variety antiserum was absorbed with *X. campestris* v. *armoraciae* (XC4). This indicated that the two organisms differ only in additional components for *X. vesicatoria* v. *raphani*, not present in *X. campestris* v. *armoraciae*. This complete unilateral absorption has been observed in other combinations, i.e., *X. taraxaci* with *X. vesicatoria* v. *raphani*, *X. papavericola* with *X. vesicatoria*, and *X. hederæ* with *X. vascularum*. In each instance the reciprocal absorption did not negate the homologous reaction.

In contrast to the results obtained in the *Xanthomonas vascularum* group are those noted with *X. phaseoli* group organisms. It is to be observed (table 3) that absorption of any antiserum of organisms in this category by any heterologous culture of the group leaves only species-specific factors. It can be assumed that the group-specific components are identical and uniform in distribution.

TABLE 2  
*Absorption of Xanthomonas vascularum group antisera by members of the group*

ORGANISM AGGLUTINATED	UNAB-SORBED	XV14 ANTISERUM ABSORBED WITH				UNAB-SORBED	XV1 ANTISERUM ABSORBED WITH				XC4 ANTISERUM ABSORBED WITH			
		XV16	XH1	XP5	XV7 or XV13		XH1	XP5	XV16	XC4	XV1	XV14	XP5	XV16
<i>X. vesicatoria</i> (XV14)	++++	++	++	++	-	++	+	++	++	+	++	-	++	-
<i>X. vesicatoria</i> v. <i>raphani</i> (XV16)	+++	-	+	+	-	++	+	+	-	-	++	-	-	-
<i>X. hederae</i> (XH1)	+++	+	-	+	-	++	-	++	+	+	++	-	-	-
<i>X. incanae</i> (X18)	++++	-	+	-	-	++	-	++	-	-	++	-	-	-
<i>X. papavericola</i> (XP5)	++++	-	++	-	-	++	+	-	+	-	++	++	-	-
<i>X. campestris</i> v. <i>armoraciae</i> (XC4)	++++	-	++	+	-	++	+	++	++	-	++	++	++	-
<i>X. vascularum</i> (XV1)	+++	-	-	-	-	++	++	++	++	++	-	-	-	-
<i>X. tarazaci</i> (XT11)	++++	-	++	+	-	++	-	++	++	-	+	++	++	-

- = no agglutination at 1:50.  
 + = agglutination at 1:50 or 1:100.  
 ++ = agglutination at 1:200 or 1:400.  
 +++ = agglutination at 1:800 or 1:1,600.  
 ++++ = agglutination at 1:3,200 or above.



Of the five organisms in this division two were found to be serologically identical. They were *X. geranii* and *X. pelargonii*. They reacted identically in all absorbing combinations and also were alike as shown by mirror absorption with their respective immune serums (table 3).

TABLE 3  
*Agglutinin absorption experiments in the Xanthomonas phaseoli group*

SERUM	ABSORBED WITH	ORGANISM AGGLUTINATED WITH				
		XG4	XP7	XP14	XM13	XP19
<i>X. geranii</i> (XG4)	—	++++*	++++	+++	+++	++
	XP7	—	—	—	—	—
	XP14	++	++	—	—	—
	XM13	+++	++	—	—	—
	XP19	++	++	—	—	—
<i>X. pelargonii</i> (XP7)	—	++++	+++	+++	+++	++
	XG4	—	—	—	—	—
	XP14	+++	+++	—	—	—
	XM13	++	+++	—	—	—
	XP19	++	++	—	—	—
<i>X. phaseoli</i> (XP14)	—	++++	++++	++++	++++	++
	XG4	—	—	++	—	—
	XP7	—	—	++	—	—
	XM13	—	—	+++	—	—
	XP19	—	—	++	—	—
<i>X. malvacearum</i> (XM13)	—	++	+++	+++	++++	+++
	XG4	—	—	—	++	—
	XP7	—	—	—	++	—
	XP14	—	—	—	++	—
	XP19	—	—	—	+++	—
<i>X. phaseoli</i> v. <i>fuscans</i> (XP19)	—	+++	++	++	+++	++++
	XG4	—	—	—	—	+++
	XP14	—	—	—	—	++
	XM13	—	—	—	—	+++

\* See table 2 for explanation of symbols.

The intermediate position of *Xanthomonas campestris* and *X. barbareae* between the *X. vascularum* and *X. phaseoli* groups is unique. It was evident from previous investigations (Elrod and Braun, 1947a), and as is indicated by table 4, that organisms of both groups reacted strongly in *X. campestris* (XC10) and *X. barbareae* (XB2) antisera. Also, these two species reacted in all of the individual serums of the two groups. There was, however, no evidence of reaction between the groups per se, when mucoid-free antigens were used (*loc. cit.*).

The similarity of reaction manifest between *Xanthomonas campestris* and *X. barbareae* led to the performing of mirror absorption tests between the two. This

resulted in complete reduction of activity in each case, indicating the serological identity of the two organisms.

Absorption of *Xanthomonas campestris* (XC10) antiserum (table 4) by any member of the *X. vascularum* division removed all of the agglutinins for this group, while reducing the components peculiar to *X. campestris*, and not removing the factors active against the *X. phaseoli* group. Likewise, absorption of *X. campestris* immune serum by members of the *phaseoli* group removed antibodies active for the latter organisms, but failed to obliterate activity for *X. campestris* and the *vascularum* group. Multiple absorption with *X. geranii* (XG4) and *X.*

TABLE 4

*Absorption experiments of Xanthomonas campestris antiserum by Xanthomonas barbareae and Xanthomonas vascularum and Xanthomonas phaseoli group organisms*

ORGANISM AGGLUTINATED	UNABSORBED	ABSORBED BY							
		XV14	XV16	XH1	XC4 and XG4	XB2	XG3	XM13	XP14
<i>X. vesicatoria</i> (XV14)	+++	—*	—	—	—	—	++	++	+
<i>X. vesicatoria</i> v. <i>raphani</i> (XV16)	++	—	—	—	—	—	++	++	++
<i>X. hederae</i> (XH1)	++	—	—	—	—	—	++	+	++
<i>X. incanae</i> (XI3)	++	—	—	—	—	—	+	++	+
<i>X. papavericola</i> (XP5)	++	—	—	—	—	—	+++	++	++
<i>X. campestris</i> v. <i>armoraciae</i> (XC4)	+++	—	—	—	—	—	++	++	++
<i>X. vascularum</i> (XVI)	+++	—	—	—	—	—	++	++	++
<i>X. taraxaci</i> (XT11)	+++	—	—	—	—	—	+++	++	+
<i>X. barbareae</i> (XB2)	++++	+++	++	++	++	—	++	++	++
<i>X. campestris</i> (XC10)	+++	+++	++	++	++	—	+++	++	++
<i>X. geranii</i> (XG4)	+++	+++	++	+	—	—	—	—	—
<i>X. pelargonii</i> (XP7)	+++	+++	++	+	—	—	—	—	—
<i>X. phaseoli</i> (XP14)	++	+++	+	+	—	—	—	—	—
<i>X. malvacearum</i> (XM13)	++	++	+	+	—	—	—	—	—
<i>X. phaseoli</i> v. <i>fuscans</i> (XP19)	++	++	+	+	—	—	—	—	—

\* See footnote to table 2 for explanation of symbols.

*campestris* v. *armoraciae* (XC4) left *X. campestris* (XC10) antiserum only specific agglutinins (table 4). The latter species is characterized by a factor common to the *phaseoli* group, one common to the *vascularum* group, in addition to species-specific components.

#### DISCUSSION

It seems apparent from the studies made in the *Xanthomonas translucens* (Elrod and Braun, 1947b) and *X. phaseoli* groups that specific antisera for many species of the genus *Xanthomonas* could easily be prepared. Even with organisms of the *X. vascularum* group, specific sera should be available by

proper absorbing combinations. The number of group-specific factors apparently vary considerably, and, likewise, their distribution would appear to produce a variety of serological patterns. It was felt inadvisable to expend the added labor on a complete antigenic analysis, or to prepare at this time specific antisera for the *X. vascularum* organisms.

It appears certain, however, that many of the well-recognized *Xanthomonas* species (based on known host range) are distinct immunologically. Several questions arise in this regard. Is this serological specificity attributable to the prolonged growth of the organism on a well-defined host? Is the ability to infect only certain hosts due to an antigenic uniqueness? Or finally, is there no correlation between the antigenic make-up of the bacterial cell and its ability to infect specific hosts?

It is interesting to conjecture that members of the genus *Xanthomonas* have evolved from a common organism present as a saprophyte either in the soil or on the external surfaces of plants. The assumption that the many xanthomonads have arisen from a common stock is not unreasonable in the light of the uniformity of physiological characteristics evident throughout the genus. At the same time the gradual (but sometimes abrupt) antigenic differences manifest between most of the recognized species lends credence to common ancestry. If a hypothetical ancestor is assumed for the genus, then it can be argued that through variation and selection of this primitive form an organism arose that was capable of invading and surviving in plant tissues, and that, through association with a host of definite structure, an antigenic mosaic peculiar to a specific organism evolved. On this assumption, a change in antigenic structure should take place along recognized lines if a species of *Xanthomonas* foreign to a given host was made to proliferate and thrive in that host. Also, if a change in antigenic structure were possible *in vitro* (similar to that recognized in the pneumococci) there should be an accompanying shift in specific host. Reid *et al.* (1942) have, in fact, made the claim that *Pseudomonas fluorescens* changed antigenically to become identical with *Phytomonas tabaci* in the "M" phase by association on clover plants during a single season. These experiments are open to criticism, however, because of the lack of rigidly controlled experimental conditions.

Not all xanthomonads are immunologically defined, assuming that all of the described species are valid. The serological identity of *Xanthomonas campestris* and *X. barbareae* is not in the least illogical. The latter organism was isolated and described by Burkholder (1941) in search for a reservoir of *X. campestris*. The xanthomonad isolated by him was declared to be a different entity from *X. campestris*. This was occasioned by the apparent inability of the two organisms to cross-infect their hosts of isolation. The description of the winter cress organism failed to distinguish it from *X. campestris*. Serologically we found the two to be identical. Inasmuch, however, as winter cress (*Barbarea vulgaris*) belongs in the family *Cruciferae*, this antigenic similarity with *X. campestris*, whose hosts number many *Cruciferae*, can be reconciled. Yet it is far more difficult to resolve the facts presented in part II of this series. In this it was pointed out that *X. begoniae* (pathogenic on begonia), *X. cucurbitae* (pathogenic on field

pumpkin), and certain strains of *X. translucens* (pathogenic on certain grasses) were serologically identical. These apparent discrepancies between pathological and serological results might be resolved if exhaustive comparative pathological studies were made. On the other hand, the ability to infect a given host may not be, and in certain instances is not, reflected in the measurable antigenic make-up of the organism. For example, *X. phaseoli* and *X. phaseoli* v. *sojense* are both capable of attacking the common bean (*Phaseolus vulgaris* L.), yet the strains of both species used in this study have no common antigenic fraction that is measurable by the method applied here.

While many of the described species show a correlation of host and serological specificity as suggested by Link and Sharp (1927), the antigenic make-up of these organisms is, in all probability, not determined by the host in which they develop. The data presented suggest, on the other hand, that these properties vary independently of the ability of the organisms to infect given host species.

#### SUMMARY

Three immunological divisions of the genus *Xanthomonas*, the *X. vascularum*, *X. phaseoli*, and *X. campestris* groups, were studied in regard to intra- and inter-group relationships.

The *Xanthomonas vascularum* group was found to be linked by a multitude of group factors. Isolation of species-specific factors in homologous immune serums were not obtained by a single heterologous absorption.

The members of the *Xanthomonas phaseoli* group had common group components. Absorption of any of the individual antiserums by a heterologous organism of the group left specific factors and removed all group components.

The two members of the *Xanthomonas campestris* group, *X. campestris* and *X. barbareae*, were found to be identical by mirror absorption. A "*campestris*" factor was evident and in excess of "*phaseoli*" and "*vascularum*" group components that characterize the *X. campestris* antigenic make-up.

The relationship of serological specificity to host specificity is discussed. It is felt that the antigenic properties of an organism vary independently with the ability to infect given host species.

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# AN ATYPICAL STRAIN OF PSEUDOMONAS AERUGINOSA

PAUL H. KOPPER

*Department of Bacteriology, The Chicago Medical School, Chicago, Illinois*

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Recently Kopper and Beard (1947) reported the isolation from human urine of a strain of *Pseudomonas* closely resembling *Pseudomonas aeruginosa* in cultural and biochemical characteristics, but differing from it in its ability to break down creatine and creatinine with the resultant formation of urea, ammonia, and carbon dioxide. The organism was grown on a medium containing 2 per cent creatinine, 2 per cent agar, and 5 per cent urine in distilled water.

Porter (1946), citing den Dooren de Jong, lists creatine and creatinine among a number of compounds which can serve as sources of nitrogen for *P. fluorescens* in a basal medium composed of 2 per cent agar, 0.1 per cent  $K_2HPO_4$ , 1.0 per cent glucose, and 1.0 per cent  $CaCO_3$  in tap water. In this study the same medium was used without agar. Upon addition of 0.1 per cent creatinine it was found to support adequately the growth of *P. aeruginosa* and *Pseudomonas fluorescens*. Three strains of the former and two of the latter species were tested. The amounts of creatinine broken down were exceedingly small. The strain isolated from urine, on the other hand, proved to be a very active fermenter of creatinine.

## EXPERIMENTAL PROCEDURE

An attempt to cultivate the variant strain in a 0.1 per cent solution of creatinine in tap water was unsuccessful. When 1.0 per cent autoclaved urine was added growth took place. A comparison of the reported analysis of Chicago tap water and the composition of urine revealed that the latter was rich in phosphates which the former lacked. A M/150 phosphate buffer solution of pH 7 prepared with  $KH_2PO_4$  and  $Na_2HPO_4$  in distilled water containing 0.1 per cent creatinine proved a suitable medium for the growth of the organism. For the buffer mixture, solutions of  $KH_2PO_4$  of pH 4.5 or of  $Na_2HPO_4$  of pH 9 could be substituted. Incubation at 37 C or at room temperature (20 to 24 C) was equally effective in promoting bacterial reproduction. In experiments reported in this study 0.1 per cent creatinine solutions in M/150 phosphate buffer of pH 7 were used and will be referred to as phosphate-buffered creatinine solutions.

Creatinine phosphate agar was prepared with 2 per cent agar and 2 per cent creatinine in a M/60 phosphate buffer solution of pH 7 in distilled water. Nutrient broth and nutrient agar were prepared with Difco neopeptone. All cultures were incubated at room temperature. Creatinine was determined with alkaline picrate. Color intensities were measured in a Cenco-Sheard photometer.

## RESULTS

*The nature of the creatinine-decomposing enzyme (creatinase). To determine the constitutive or adaptive character of the creatinine-decomposing enzyme of*

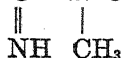
the variant strain, cultures were transferred in series into nutrient broth at 24-hour intervals. Twenty transfers were made. After each transfer the presence of creatinase was ascertained by inoculating a loopful of the nutrient broth culture into phosphate-buffered creatinine solution. The enzyme activity was not lost, but declined gradually as manifested in a delay of growth for 48 hours and in its limitation mainly to the surface of the medium.

Three stock culture strains of *P. aeruginosa* and two stock culture strains of *P. fluorescens* failed to grow in phosphate-buffered creatinine solution. An attempt was made to adapt one of the strains of *P. aeruginosa* to this medium. The organism was inoculated into nutrient broth containing 0.2 per cent of creatinine and was transferred in series at 24-hour intervals for 12 successive days. No measurable breakdown of creatinine took place, nor did growth occur in phosphate-buffered creatinine solution inoculated from the last one of the 12 serial transfers.

In another experiment successive transfers in decreasing amounts of nutrient broth, brought up to volume with distilled water, were made. The creatinine concentration was kept constant at 0.2 per cent. A method of bacterial adaptation described by Hegarty (1939) was used. Transfers were made in series every 90 minutes, at which time the end of the lag phase of *P. aeruginosa* was assumed to have been reached. All tubes were inoculated heavily. Six serial transfers were made. Tests for creatinase activity of the bacteria present in the last tube gave negative results.

Finally a heavy inoculum of the stock culture strain was streaked on several plates of creatinine phosphate agar medium and incubated at room temperature for 2 weeks. No growth occurred. It would seem that the strain used was not adaptable to creatinine under the experimental conditions outlined.

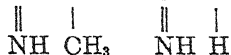
*Enzyme specificity and growth requirements.* Kopper and Beard (1947) observed that the creatinine-decomposing enzyme of the atypical strain acted on creatinine, creatine, and glycocyamidine, but failed to attack hydantoin. Phosphate-buffered solutions containing 0.1 per cent creatine, glycocyamidine, and hydantoin, respectively, were prepared and inoculated with the organism. Growth developed in creatine but not in glycocyamidine or hydantoin. In order to determine a possible cause for the discrepancy between the action of the enzyme on glycocyamidine and the failure of the strain to reproduce on this substrate, an attempt was made to grow the organism on the hydrolytic products of creatinine and glycocyamidine. These two chemical compounds are internal anhydrides of creatine ( $\text{NH}_2\text{·C} \cdot \text{N·CH}_2\text{·COOH}$ ) and glycocyamine ( $\text{NH}_2\text{·C}$



$\cdot\text{NH·CH}_2\text{COOH}$ ), respectively. The products of the hydrolysis of creatine would be urea and sarcosine, of glycocyamine urea and glycine. Phosphate-buffered solutions of 0.1 per cent urea, sarcosine, and glycine, respectively, were prepared and inoculated with cultures of the atypical strain and of three strains of *P. aeruginosa* and two strains of *P. fluorescens*. No growth took place in urea. Sarcosine supported adequately the growth of all



strains. Glycine proved to be a poor medium. The organisms either failed to multiply in it or did so only slightly after prolonged incubation. This would seem to present additional evidence for the hydrolytic action of the creatinine-decomposing enzyme of the atypical strain. The enzyme may effect the splitting of C · N and C · N linkages, but growth can only be supported by the



resulting split products. On a good nutrient such as sarcosine the organisms multiply readily, which leads to the production of more enzyme, which in its turn causes a further breakdown of creatinine or creatine, whichever is the substrate, and a greater accumulation of sarcosine. On a poor nutrient such as glycine, on the other hand, reproduction is so slow that no proper chain reaction can develop, which may account for the inadequacy of glycohydrazide as the sole source of carbon and nitrogen in a culture medium for the growth of the atypical strain.

*Preservation of cultures of the atypical strain.* Cultures of the atypical strain were kept on creatinine phosphate agar slants at room temperature. When bacteria were transferred from such slants to phosphate-buffered creatinine solution or nutrient broth after 8 to 10 days, they failed to grow. The organisms lost their viability also on nutrient agar, to which 2 per cent creatinine had been added, within the same length of time. Their creatinase activity, however, was unaffected, as shown by the disappearance of creatinine from solutions incubated with suspensions of the dead organisms. Both viability and creatinase activity could be preserved by keeping cultures on nutrient agar slants aerobically or on creatinine phosphate agar slants under oil. This was proved by transfers from such slants after 45 and 60 days, respectively. Work is now in progress to investigate the cause of the delayed lethal effect of creatinine agar media on cultures of the atypical strain under aerobic conditions.

#### DISCUSSION

Karström's differentiation of bacterial enzymes into "constitutive" and "adaptive" enzymes was enlarged upon by Krebs and Eggleston (1939), who subdivided the latter into "partially adaptive" enzymes, which are formed in the absence of the specific substrate but increased in its presence, and "totally adaptive" enzymes, which are formed only in the presence of the specific substrate. The creatinine-decomposing enzyme described by Dubos and Miller (1937) was shown to be "totally adaptive." Evidence presented in this study would favor the classification of the creatinase of the atypical strain of *P. aeruginosa* as a "partially adaptive" enzyme. One can only speculate on the mode of origin of such enzymes. They may arise from mutations of the parent strain, which are of a more fundamental character than those producing "totally adaptive" enzymes. This would explain the greater difficulties encountered in inducing them.

As pointed out by Luria (1947), most bacterial classifications are only determinative keys, which cannot be compared with the well-defined systems of zoological and botanical taxonomy. Many bacterial species and even genera

are separated on the basis of character differences that may be brought about by a single mutational step. The organism described here may have arisen in such a way, since aside from its creatinase activity it is indistinguishable from the species *P. aeruginosa*.

#### SUMMARY

A strain of *Pseudomonas aeruginosa*, first isolated from urine, was cultivated in a phosphate-buffered solution containing creatinine as the sole source of carbon and nitrogen.

The strain possesses a specific creatinine-decomposing enzyme, creatinase, which is not lost after 20 successive transfers through nutrient broth without creatinine.

Attempts to adapt a stock culture strain of *P. aeruginosa* to grow in a phosphate-buffered creatinine solution were unsuccessful.

The creatinase acts on glycohydrazide, but this compound is inadequate for serving as the sole source of carbon and nitrogen for the growth of the atypical strain.

Sarcosine, a hydrolytic product of creatinine and creatine, represents a good culture medium for the atypical strain and five other strains of *P. aeruginosa* and *Pseudomonas fluorescens* tested. Glycine, a hydrolytic product of glycohydrazide, is a poor nutrient for all strains.

Cultures of the strain could be preserved on nutrient agar aerobically or on 2 per cent creatinine phosphate agar under oil for 45 and 60 days, respectively.

Cultures kept aerobically on creatinine phosphate agar or on 2 per cent creatinine nutrient agar lost their viability but not their creatinase activity within 8 to 10 days.

The nature of the enzyme and its possible mode of origin are discussed.

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# THE CORRELATION BETWEEN THE INHIBITION OF DRUG RESISTANCE AND SYNERGISM IN STREPTOMYCIN AND PENICILLIN<sup>1</sup>

MORTON KLEIN AND LEONARD J. KIMMELMAN

Department of Bacteriology, School of Medicine, University of Pennsylvania,  
Philadelphia 4, Pennsylvania

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The problem of drug resistance has become an important limiting factor in the therapeutic efficiency of streptomycin (Buggs *et al.*, 1946; Finland *et al.*, 1946; Bondi *et al.*, 1946). We have previously shown in the case of streptomycin that of 13 strains tested all had the ability to throw off, spontaneously, variants resistant to streptomycin (Klein and Kimmelman, 1946; Klein, 1947). The destruction by streptomycin of the mass of susceptible bacteria and the multiplication of the few highly resistant variants was indicated to be a mechanism for the development of streptomycin resistance. Alexander and Leidy (1947), working with *Hemophilus influenzae*, have recently obtained similar results. Clinically, the inhibition of the rapid development of streptomycin resistance may then require the destruction of a relatively small number of resistant bacteria, which might be effected by the addition of a low concentration of another drug. In the present work we have therefore studied the combined action of streptomycin, penicillin, and sulfadiazine *in vitro* and determined the relationship between the synergistic action of the compounds and the inhibition of the development of streptomycin resistance.

## MATERIALS AND METHODS

*Staphylococcus aureus*, susceptible to streptomycin, penicillin, and sulfadiazine, was used as the test organism. A casein hydrolyzate medium (Strauss, Dingle, and Finland, 1941) containing 0.5 per cent glucose provided a clear medium which was convenient in the determination of growth rates turbidimetrically in the Klett-Summerson photoelectric colorimeter. The presence of the glucose resulted in a drop in pH after 24 hours that did reduce the streptomycin activity (Geiger, Green, and Waksman, 1946). However, this did not interfere with the interpretation of the results on the combined drug action.

The tests for drug activity were performed as follows: Six ml of the casein hydrolyzate medium, containing the various drugs singly or in combination, were added to the Klett-Summerson tubes, and a standard inoculum of 0.1 ml of a 20- to 24-hour culture, diluted to give a reading of 50 on the Klett-Summerson colorimeter (approximately 15,000,000 bacteria), was seeded into each of the tubes. This large inoculum provided a rapid initial growth, which permitted the taking of turbidity readings at 6 hours, in addition to the 12-, 24-, and 48-hour readings. In preliminary assays it was found that the 24-hour growth

<sup>1</sup> This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

curves obtained on the basis of turbidity readings followed essentially the same pattern as the 24-hour growth curves obtained on the basis of viable counts. The 48-hour readings were included to show any delayed growth obtained with the inhibitors.

The increase in drug resistance was estimated after 48 hours' incubation at 37 C. The bacteria were subcultured from the initial drug assays after 48 hours and grown for 20 to 24 hours in the casein hydrolyzate medium. A standard 0.1-ml sample was inoculated into the same drug concentration initially used, and growth was again determined turbidimetrically. The increase in the growth rate was then a measure of the increase in resistance to the drug. Turbidity readings of the medium, plus the standard inoculum, were taken at the beginning of all experiments, and the increase in turbidity over the initial reading was recorded. Only increases in turbidity greater than a reading of 10 were recorded in the graphs, and the turbidity readings were plotted as ordinates on a log scale. All growth curves are representative experiments from at least four separate assays.

#### RESULTS

In figure 1 are shown the growth rates of the initially susceptible bacteria grown in partially inhibitory concentrations of penicillin, streptomycin, and sulfadiazine. The increase in resistance to each of the drugs is indicated for the 48-hour subcultures reassayed against the same concentration of the respective drugs. The bacteria subcultured after 48 hours from the initial assays of each of the drugs, and retested against the same concentration of each drug, showed a sharp increase in streptomycin resistance, a moderate increase in penicillin resistance, and no increase (frequently a slight decrease) in the rate of growth in the presence of sulfadiazine. The increase in resistance after 48 hours to one drug did not result in an increase in resistance to any of the other drugs.

We have found that the rate of increase in resistance to penicillin and streptomycin is a function not only of the specific drug but of the concentration of the drug used. It was found that the greater the partially inhibitory action of penicillin or streptomycin, the greater the increase in resistance. When bacteria were grown in 2 units of streptomycin per ml, subcultured after 48 hours, and retested against 8 units of streptomycin, they showed only a relatively small increase in resistance. Bacteria grown in 4 and 8 units of streptomycin per ml showed significantly greater increases in resistance. Likewise, bacteria subcultured after 48 hours' growth in 0.02 of a unit of penicillin per ml and retested against 0.06 units of penicillin per ml showed only a slight increase in resistance when compared with the increase in penicillin resistance of bacteria grown in 0.04 and 0.06 units of penicillin per ml. This role of drug concentration in the development of penicillin and streptomycin resistance can be explained on the basis of the selection and multiplication of resistant variants. In high concentrations of penicillin or streptomycin which did not completely inhibit growth, all but a few of the most resistant bacteria in the initial inoculum would be eliminated. These few bacteria could multiply and resistant variants would be

thrown off in the direction of greater drug resistance. However, at lower drug concentrations one would not obtain so effective a selection of the few resistant variants; more of the less resistant bacteria would survive and on subculture they would tend to overgrow the few most resistant variants. Upon reassaying, such a culture would show only a moderate or slight increase in resistance. We also observed that when there was no significant inhibition by

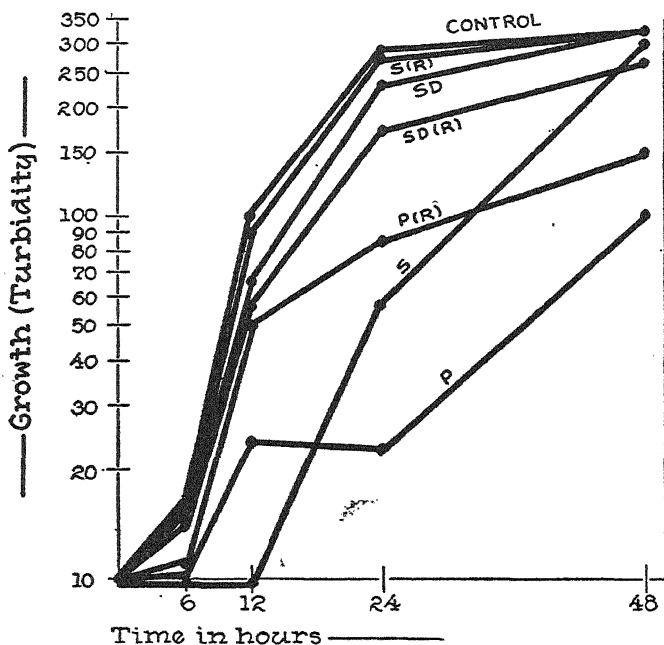


FIG. 1. DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN, PENICILLIN, AND SULFADIAZINE (*STAPHYLOCOCCUS AUREUS*)

Medium: casein hydrolyzate, pH 7.4. Inoculum: 0.1 ml of 20- to 24-hour culture (approx. 15,000,000 organisms). P = penicillin, 0.06 u/ml seeded with susceptible bacteria. S = streptomycin, 8.0 u/ml seeded with susceptible bacteria. P(R) = penicillin, 0.06 u/ml seeded with organisms grown for 48 hours in 0.06 u/ml penicillin. S(R) = streptomycin, 8.0 u/ml seeded with organisms grown for 48 hours in 8.0 u/ml streptomycin. SD(R) = sulfadiazine, 1:5,000 seeded with organisms grown for 48 hours in 1:5,000 sulfadiazine.

the drug, i.e., when no selection of the more resistant forms would occur, there was no demonstrable increase in resistance.

*Combined action of two drugs and the inhibition of streptomycin resistance.* We determined the relative effectiveness of sulfadiazine and penicillin when added to streptomycin both with respect to their ability to increase the inhibitory action of streptomycin and their effectiveness in decreasing the streptomycin resistance of bacteria surviving the action of the drug. The results are shown in figures 2 and 3. When 1:5,000 sulfadiazine or 0.06 of a unit of penicillin were added to 8 units of streptomycin, the combined action of the two drugs was

greater than either drug alone and the effect was not a simple additive one. Sulfadiazine in a 1:5,000 concentration, which was less inhibitory than 0.06 units of penicillin, was far more effective when combined with streptomycin than was penicillin, and this greater effectiveness of sulfadiazine as a synergist was related to its ability to reduce more effectively the resistance of bacteria surviving the action of streptomycin. In figure 3 is shown the increase in resistance of bacteria surviving the action of (1) 8 units of streptomycin, (2) 8 units of streptomycin plus 0.06 units of penicillin, and (3) 8 units of streptomycin plus 1:5,000 sulfadiazine. The bacteria grown in streptomycin alone showed a

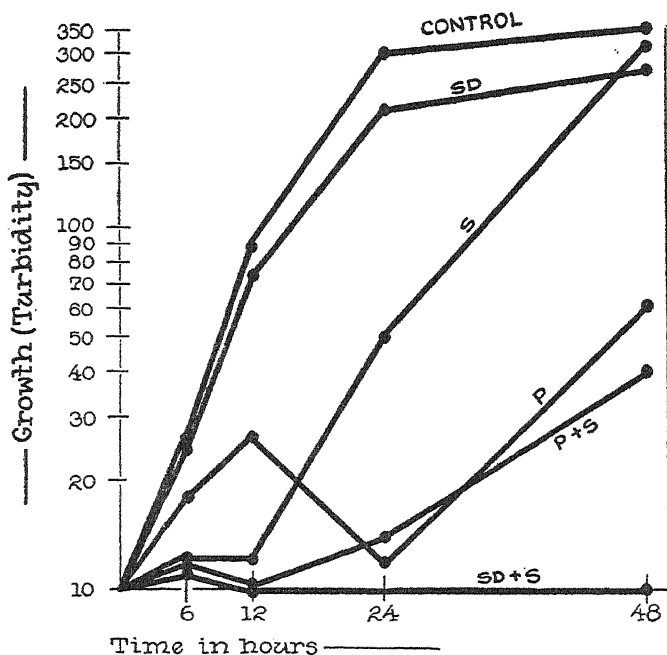


FIG. 2. INHIBITORY ACTION OF STREPTOMYCIN, PENICILLIN, AND SULFADIAZINE—SINGLY AND COMBINED—ON SUSCEPTIBLE STAPHYLOCOCCUS AUREUS

P = penicillin, 0.06 u/ml. S = streptomycin, 8.0 u/ml. SD = sulfadiazine, 1:5,000. P + S = penicillin, 0.06 u/ml plus streptomycin, 8.0 u/ml (final concentrations). SD + S = sulfadiazine, 1:5,000 plus streptomycin, 8.0 u/ml (final concentrations).

very marked increase in resistance, and the bacteria grown in the combination of streptomycin and penicillin showed almost the same increase in streptomycin resistance. However, the bacteria grown in streptomycin and sulfadiazine showed only a moderate increase in streptomycin resistance, indicating that the greater activity of sulfadiazine as a synergist was associated with a greater activity in inhibiting the development of streptomycin resistance. There was, however, in all cases an increase in streptomycin resistance over the initial susceptibility of the bacteria.

When two drugs are combined, each inhibits the development of drug re-

sistance to the other. Streptomycin was found to inhibit effectively the development of penicillin resistance, as did sulfadiazine. No increase in sulfadiazine resistance was ever observed after 48 hours when subcultures were tested from sulfadiazine alone or in combination with other drugs.

*Combined action of three drugs.* The combined action of 1:10,000 sulfadiazine, 0.04 units of penicillin, and 4 units of streptomycin was determined against *S. aureus*, and the results are shown in figure 4. The combination of streptomycin and sulfadiazine or the combination of streptomycin and penicillin effected

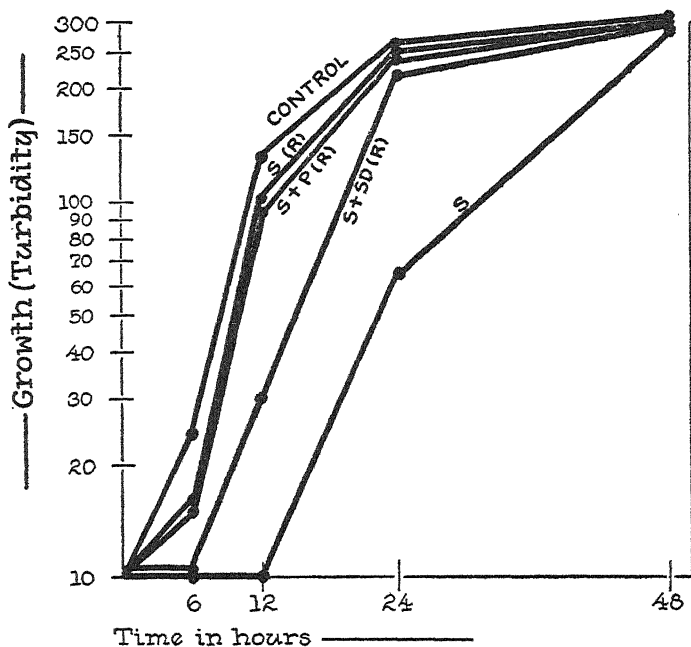


FIG. 3. INCREASE IN STREPTOMYCIN RESISTANCE OF BACTERIA PREVIOUSLY GROWN IN STREPTOMYCIN, STREPTOMYCIN-PENICILLIN, STREPTOMYCIN-SULFADIAZINE (STAPHYLOCOCCUS AUREUS)

S = streptomycin, 8.0 u/ml seeded with susceptible bacteria. S(R) = streptomycin, 8.0 u/ml seeded with organisms grown for 48 hours in 8.0 u/ml streptomycin. S + P(R) = streptomycin, 8.0 u/ml seeded with organisms grown for 48 hours in 8.0 u/ml streptomycin plus 0.06 u/ml penicillin (final concentrations). S+SD(R) = streptomycin, 8.0 u/ml seeded with organisms grown for 48 hours in 8.0 u/ml streptomycin plus 1:5,000 sulfadiazine (final concentrations).

only a partial inhibition of growth, whereas the combination of all three drugs completely inhibited growth. Though inhibition was complete, there were always a few bacteria surviving the combined drug action. These bacteria when subcultured and reassayed against streptomycin, penicillin, and sulfadiazine, respectively, never showed any increase in resistance and regularly showed a slight decrease in growth rate in the presence of streptomycin and occasionally a slight decrease in resistance to penicillin and sulfadiazine. The absence of any increase in resistance can be interpreted as being due to the

prompt inhibition of all multiplication by the three drugs with the subsequent inability of resistant variants to arise. The few surviving bacteria can be considered as nondividing cells in a physiological state temporarily unaffected by the action of the drugs.

When the three drugs were combined in lower concentrations which permitted some multiplication, e.g., penicillin 0.02 units per ml, streptomycin 4 units per ml, and 1:15,000 sulfadiazine, the bacteria when reassayed after 48 hours showed neither an increase nor a decrease in resistance. It should be pointed out again that lowering the test concentration of penicillin or streptomycin is in itself a

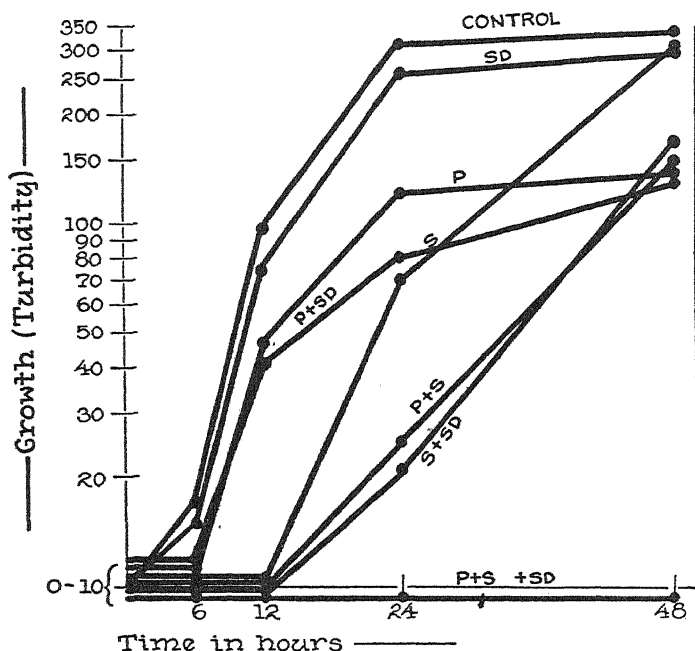


FIG. 4. INHIBITORY ACTION OF PENICILLIN, STREPTOMYCIN, AND SULFADIAZINE SINGLY AND IN COMBINATION ON SUSCEPTIBLE STAPHYLOCOCCUS AUREUS

P = penicillin, 0.04 u/ml. S = streptomycin, 4.0 u/ml. SD = sulfadiazine, 1:10,000. P+S = penicillin, 0.04 u/ml plus streptomycin, 4.0 u/ml (final concentrations). P+SD = penicillin, 0.04 u/ml plus sulfadiazine, 1:10,000 (final concentrations). S+SD = streptomycin, 4.0 u/ml plus sulfadiazine, 1:10,000 (final concentrations). P+S+SD = penicillin, 0.04 u/ml plus streptomycin, 4.0 u/ml plus sulfadiazine, 1:10,000 (final concentrations).

factor in effecting a decrease in the rate of development of drug resistance. However, this reduction in drug concentration is not in itself sufficient to eliminate completely the development of drug resistance in the case of the individual compounds.

It should be pointed out that both drugs must be present in concentrations which are in themselves inhibitory. We have found that if one exposes a streptomycin-resistant culture to streptomycin and sulfadiazine, or streptomycin and penicillin, one obtains the inhibitory action of the sulfadiazine or penicillin alone.



## DISCUSSION

The relationship between synergism and drug resistance has been previously indicated by us in a report on the combined action of penicillin and the sulfonamides (Klein and Kalter, 1945). It was found that an important factor in the observed synergism was the ability of a small amount of an added drug, in this case the sulfonamide, to prevent the multiplication of the few bacteria resistant to the test concentration of penicillin.

Several factors may be considered in the present inhibition of drug resistance resulting from the simultaneous use of several drugs. We have already indicated that the use of lower concentrations of each drug is an important factor in the decreased resistance to the drug. Carpenter, Bahn, Ackerman, and Stokinger (1945) found that when bacteria were grown in sulfathiazole, rivanol lactate, promin, and penicillin, drug resistance did not develop against any of the compounds. In the combination of four drugs Carpenter and his coworkers used one-fourth the drug concentration initially used in the development of resistance to each agent. It would be of interest to know to what extent this reduction in the concentration of the individual drugs was related to the elimination of resistance when all four drugs were combined.

As a synergist with streptomycin, the greater activity of sulfadiazine as compared with penicillin may be related to the very high degree of sensitivity of sulfadiazine to the total number of bacteria present. We have found, for example, that a 1,000-fold decrease in the size of our *S. aureus* inoculum increased the sulfadiazine titer over 30-fold, but under similar conditions the penicillin titer was increased only 3-fold. Hence when only a small number of streptomycin-resistant cells are present, low concentrations of sulfadiazine would be particularly effective.

If one assumed that a drug had an all or none effect, i.e., it either inhibited a bacterium from dividing or left the cell essentially unaltered, then the combined effect of the drugs could be explained exclusively in terms of this independent action. A given concentration of streptomycin would therefore destroy all but a small number of bacteria completely resistant to it and the small concentrations of the added drug or drugs would independently inhibit the small number of surviving bacteria. If, however, a drug can significantly modify cellular metabolism though not inhibit cell division, then it is possible that two drugs acting on a single cell may together effect complete inhibition or killing when each alone could not (Mudd, 1945). One would then have in addition to the independent action, which must occur, this combined action on a single cell.

Apart from any consideration as to the precise mode of action and with due regard to possible toxic effects, one can state that drugs having some limited degree of action against streptomycin-resistant bacteria are potential tools for reducing or eliminating the development of streptomycin resistance. It may be mentioned that antibodies and phagocytes should play a role in inhibiting the development of resistance by suppressing the multiplication of resistant cells. It is of particular interest to note that Schnitzer, Lafferty, and Buck (1946) found that drug resistance of the trypanosomes developed most rapidly in those treated experimental animals in which there was little antibody activity.

## SUMMARY

After 48 hour's growth in a casein hydrolyzate medium containing streptomycin, penicillin, or sulfadiazine, *Staphylococcus aureus* showed a marked increase in resistance to streptomycin and penicillin and no increase in resistance to sulfadiazine.

The greater the partially inhibitory concentration of streptomycin or penicillin the greater the increase in the rate of development of resistance.

Sulfadiazine, when added to streptomycin broth, was far more effective as a synergist and inhibitor of streptomycin resistance than was penicillin.

Low concentrations of streptomycin, penicillin, and sulfadiazine when combined were highly effective in inhibiting multiplication and prevented the development of drug resistance.

The results are interpreted on the basis of the selection and inhibition of resistant variants.

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# ORAL IMMUNITY TESTS OF DYSENTERY ANTIGEN IN WHITE MICE

H. M. POWELL AND W. A. JAMIESON

*Lilly Research Laboratories, Indianapolis, Indiana*

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A current publication by Cooper and Keller (1947) on oral dysentery immunity in mice prompts us to submit some results on the same subject. Slightly different bacillary preparations were used.

The present report gives results we have obtained by applying the Grasset technique to dysentery bacilli, including Shiga, Hiss, Flexner, Sonne, and Mt. Desert types. Grasset (1939) prepared typhoid "endotoxoid" by repeatedly freezing and thawing typhoid bacillus suspensions, and subsequently treating the resultant lysate with formalin to reduce the toxicity. Human doses of three or four times the usual size may be given with little discomfort, and a superior degree of immunity is claimed. Using the same technique, we have found that formalin treatment of repeatedly frozen and thawed dysentery bacillus lysate for about a month decreased the toxicity considerably as judged by intraperitoneal tests in mice. Table 1 indicates a fourfold or more (possibly 32-fold) decrease in toxicity in 26 days of formalinization at 37 C.

Following such detoxification with formalin, the culture lysates, either as single types or equal part mixed types, were usually adsorbed on starch and desiccated so that 1 gram represented 60,000 million original bacilli. The technique for oral immunity tests was that previously described (Powell, 1942) but modified by the use of white mice to assay the potency of this antigen. Following oral immunization of white mice, decimal dilutions of 18-hour agar slant cultures suspended in a fluid containing 5 per cent gastric mucin and 0.2 per cent agar were given intraperitoneally for challenge to both treated and control mice. Since the virulence of the cultures for control mice was from  $10^{-8}$  to  $10^{-10}$  part of an agar slant, there was ample opportunity to detect active immunization of test mice with infecting doses of culture much less than primarily toxic doses.

Preliminary experiments with different doses of single type and mixed type antigens, both in moist and dry condition, showed that (a) both homologous and slight heterologous type immunity can be produced orally in white mice; (b) antigen derived from 20,000 million bacilli appeared to be the best oral dose, being sufficiently strong to incite immunity and without harmful action on mice—10,000 million bacilli appeared insufficient and 50,000 million appeared somewhat toxic; (c) 10 oral doses, each derived from 20,000 million bacilli, given to mice in 5 days, i.e., 2 doses per day, sufficed at 1 week after the last dose to incite immunity against 1, 10, and sometimes 100 MLD of culture suspended in the mucin-agar enhancement fluid; and (d) 20 doses of half-size were the equivalent of (c). The degree of oral immunity attained here is quantitatively

about the same as that observed in mice by Felsen and Osofsky (1938) with injectable vaccine, and successful immunization against great multiples of a fatal dose of living dysentery bacilli has not been reported.

A lot of antigen representing 12,000 million bacilli of each of the five types referred to above, or a total of 60,000 million bacilli per gram in starch, has been tested orally in 95 white mice against the five types of infections. On account of the bulkiness of the starch vehicle we used the sequence of doses indicated under (d) above. The results of these tests are recorded in table 2. The various

TABLE 1  
*Detoxification of dysentery bacillus antigen*

INTRAPERITONEAL MOUSE DOSE 0.5 ML	FORMALINIZED ANTIGEN INCUBATED AT 37 C (DAYS)				
	0	5	12	19	26
Undiluted	1*	1	1	1	5
Diluted					
1:2	1	1	1	1	4
1:4	2	S	4	4	5
1:8	1	3	4	4	6
1:16	3	5	4	S	S
1:32	2	S	S	S	S

\* Legend: each figure indicates day of death of a mouse; S indicates survival at 7 days.

TABLE 2  
*Oral immunity tests of dysentery antigen in white mice against five types of dysentery bacilli*

FRACTION OF A SLANT OF DYSEN- TERY CULTURE IN- JECTED IN 0.5 ML	SHIGA		HISS		FLEXNER		MT. DESERT		SONNE	
	Immun- ized	Controls	Immun- ized	Controls	Immun- ized	Controls	Immun- ized	Controls	Immun- ized	Controls
10 <sup>-5</sup>	DDDS	—	DDD	—	—	—	—	—	DDDS	—
10 <sup>-6</sup>	DDDD	DDD	DDD	DDD	—	—	—	—	DDSS	DDD
10 <sup>-7</sup>	DSSS	DDD	DDSD	DDD	DD	DD	DS	DD	DDSSS	DDD
10 <sup>-8</sup>	SSSS	DDD	DDD	DDD	DD	DD	DD	DD	DDSSS	DDS
10 <sup>-9</sup>	—	SSS	DDSD	DDS	DDSD	DDSD	DDSD	DDD	—	SSS
10 <sup>-10</sup>	—	—	SSS	—	SS	DD	DDSD	DDDD	—	—

Legend: D = mouse dead within 3 days; S = mouse surviving; — = test not done.

test cultures, in decimal dilution in the mucin-agar virulence enhancement fluid, exhibited a high degree of virulence for normal control mice. Comparison in immunity between groups of treated and control mice injected with each of the series of live culture dilutions may be made in the horizontal columns in the table. Considerable immunity is exhibited toward the Shiga and Sonne types, and weaker immunity is exhibited against the Hiss, Flexner, and Mt. Desert types. A higher bacterial "count" in the mixed type vaccine is difficult to use owing to the over-all residual toxicity.

It is concluded that dysentery antigen, after repeated freezing and thawing, may be detoxified considerably with formalin, and oral assay of this antigen can be done in a period of 5 days of immunization, 7 days of waiting, then 2 or 3 days for completing live-culture tests, or about 2 weeks in all. The oral response in mice appears to be better against the Shiga and Sonne types than against the Hiss, Flexner, and Mt. Desert types. Possibly variable doses of the different types of organisms in the vaccine might have improved the results.

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# PRODUCTION OF ASPERGILLIC ACID BY SURFACE CULTURES OF ASPERGILLUS FLAVUS

CARL R. WOODWARD, JR.

*Bacteriological Production Laboratories of E. R. Squibb & Sons, New Brunswick, N. J.*

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It has been reported by White (1940), Rake, McKee, and Jones (1942), White and Hill (1943), Jones, Rake, and Hamre (1943), and Bush, Dickison, Ward, and Avery (1945) that the fungus *Aspergillus flavus* produces an antibiotic substance known as aspergillic acid. *Aspergillus flavus* is also known to produce other antibiotic substances such as flavacidin (McKee, Rake, and Houck, 1943; McKee and MacPhillamy, 1943), and flavicin (Bush, Goth, and Dickison, 1945). However, for economical large-scale production of aspergillic acid, it was necessary to try additional media and methods of cultivation. The studies described in this report resulted in the development of suitable methods for obtaining valuable increases in the yields of material produced by the fungus.

## METHODS OF CULTIVATION

White and Hill reported yields of 0.005 to 0.07 mg of crude crystalline material per ml of medium when *Aspergillus flavus* grew in surface culture at 23 C on a solution containing 2 per cent tryptone and 0.5 per cent sodium chloride. Rake *et al.* reported yields of 0.1 to 0.25 mg of crystalline aspergillic acid per ml of medium of the same composition. Bush *et al.* reported yields of 0.3 mg of crude crystalline material per ml of a solution containing 2 per cent Difco peptone and 2 per cent lactose. Similar results were obtained when the same media and methods were tried in this laboratory.

In the attempt to increase the yields in this laboratory several modified medium formulas were tried. Some were promising but others gave completely or nearly completely negative results. Individual media containing suitable sources of necessary nutrients such as soybean meal, vegetable meal, casamino acid, veal broth, Czapek-Dox, neopeptone, corn steep liquor, Brewer's yeast, and proteose peptone produced no detectable amount of aspergillic acid. A few other media which contained boiled potatoes, *dl*-isoleucine, Sabouraud's solution, or brain-heart infusion as the essential ingredient produced substantial amounts of aspergillic acid, but the one which gave the best yield as determined by assay was a simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol. This medium on the average yielded 0.8 mg of aspergillic acid per ml of solution in actual large-scale production lots and assayed over 1 mg per ml in the case of some smaller, experimental lots.

## EXPERIMENTAL RESULTS

Fifty ml of the yeast extract glycerol medium were sterilized per 250-ml Erlenmeyer flask at 15 pounds for 15 minutes. The initial pH range was 6.3 to

6.6. The inoculum was  $10^{10}$  spores in 1 ml of spore suspension. Incubation was at 25 C. These experiments represent several dozen flasks—each individual flask having been assayed biologically and spectrophotometrically.<sup>1</sup>

After inoculation of a flask, growth commenced promptly and by 48 hours a heavy, white, wrinkled pellicle was formed. The liquid under the pellicle was tested for activity and pH daily from the end of the third day until the twelfth day. The results of these experiments are shown in figures 1, 2, and 3.

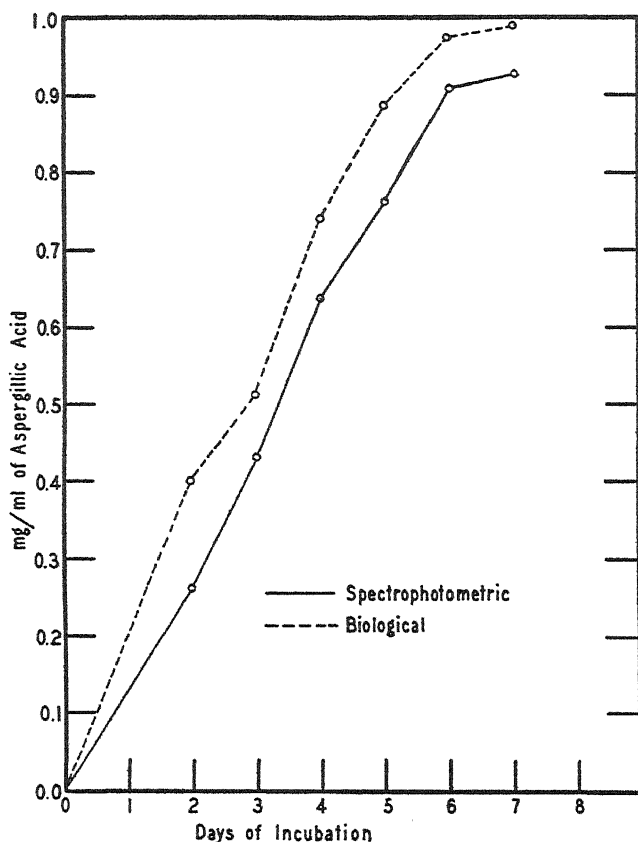


FIG. 1. ASPERGILLILIC ACID—ASSAY VS. DAYS

It will be noted from the graphs that as the pH rises the activity also increases, and on the average the optimum incubation period is 6 to 7 days. The rise in pH is fairly rapid and consistent. The peak activity, as a rule, is obtained when the pH reaches 7.8. The greatest rise in pH occurs during the first two days as the pellicle is forming, and thereafter rises more slowly.

<sup>1</sup> Similar results were obtained in 110 production lots of 200 one-gallon bottles, each containing approximately 300 ml medium. As each production lot was harvested after 7 days' incubation, it was pooled and assayed as such both biologically and spectrophotometrically.



## METHOD OF ASSAY

Previously, Rake *et al.* (1942, 1943) reported a rapid test for the activity of certain antibiotic substances, including aspergillic acid, based on the interference with the luminescence produced by luminescent bacteria. This interference can be directly correlated with antibacterial activity. However, in this laboratory two other methods were preferred: the spectrophotometric method which

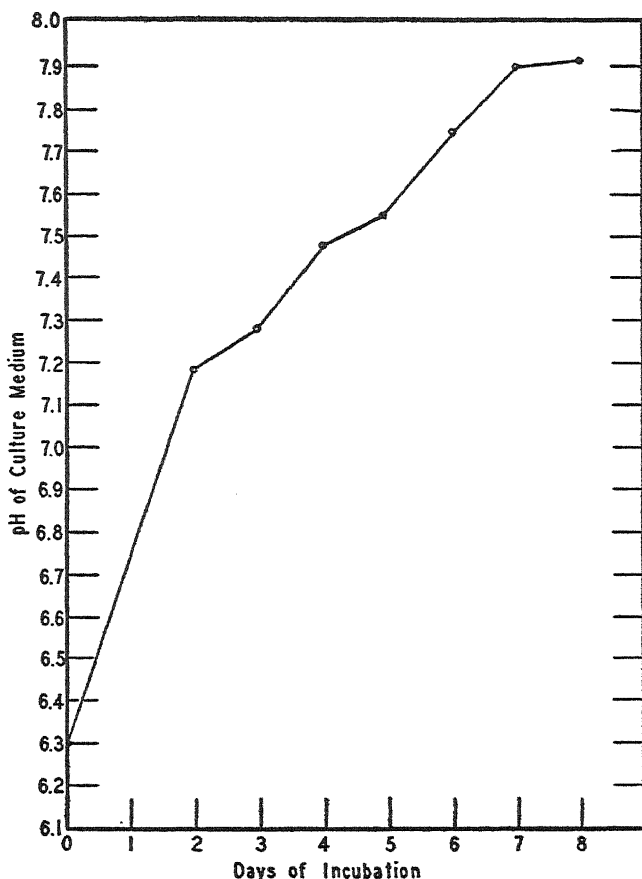


FIG. 2. ASPERGILLIC ACID, pH CURVE

is based on the ultraviolet absorption curve for aspergillic acid (maximum at 336  $m\mu$  in buffer), and the biological method in which activity is tested against a standard solution of aspergillic acid (serial tube dilution). The organism used in the latter test is the Heatley strain of *Staphylococcus aureus*.

The two assays confirm each other (Dr. J. D. Dutcher, to be published). Spectrophotometrically, it is impossible to differentiate between aspergillic acid and hydroxy-aspergillic acid. Biologically the two acids are completely dif-

ferent, the hydroxy-aspergillic acid being inactive, whereas the aspergillic acid is quite active. Physically and chemically the precipitated materials are not alike. Hydroxy-aspergillic acid has a melting point of 149 to 150 C and has 3 atoms of oxygen in its chemical structure, whereas aspergillic acid melts at 90 to 100 C and has but 2 atoms of oxygen in its chemical structure.

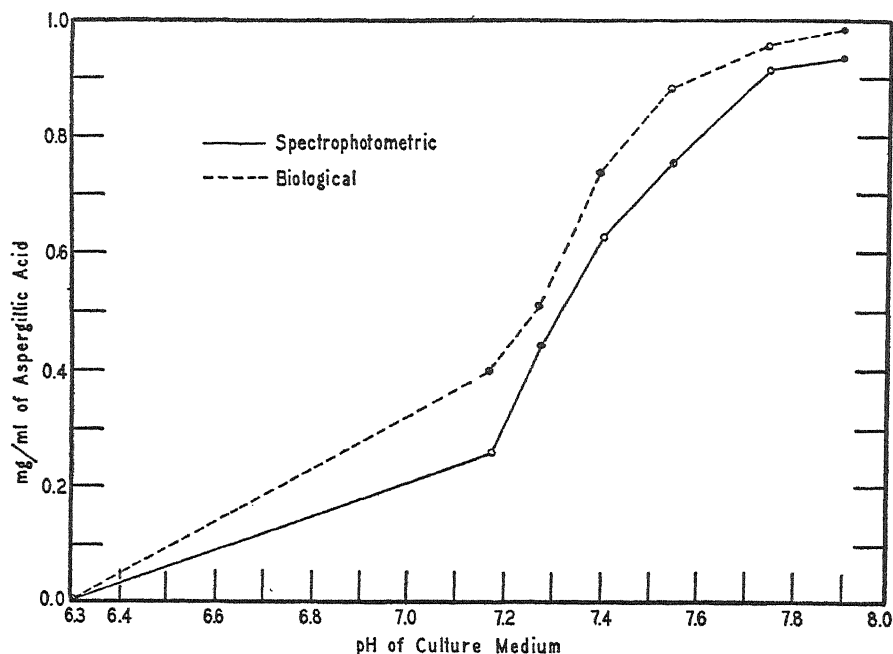


FIG. 3. ASPERGILLIC ACID—ASSAY VS. PH

TABLE 1

*The effect of yeast on the yield of aspergillic acid*

YEAST NO.	INITIAL PH	FINAL PH	SPECTRO-ASSAY	BIO-ASSAY
			mg/ml	mg/ml
333607	6.55	8.8	1.01	0.76
380588	6.5	8.9	0.03	0.68
378856	6.4	8.5	0.07	0.81
382370	6.5	8.75	0.85	0.60
382097	6.5	8.5	0.92	0.80

If a high titer in crude broth is obtained biologically, this must be verified spectrophotometrically, since *Aspergillus flavus* also produces penicillinlike substances. If a high titer in crude broth is obtained spectrophotometrically, this must be verified biologically to make certain that hydroxy-aspergillic acid is not being produced. If a high titer is obtained in both the chemical and bio-assay, a relatively large amount of aspergillic acid may be expected in the extraction or isolation process.

It is a relatively simple matter to produce hydroxy-aspergillic acid, but aspergillic acid is more difficult to produce. Experience has shown that not all lots of yeast extract will produce a high quantity of aspergillic acid.

Several lots of Difco yeast extract were tested and from the results in table 1 it can be seen that production of aspergillic acid depends on the yeast that is in the medium.

Yeasts 333607, 382370, and 382097 yielded the greatest amount of aspergillic acid, while yielding a small percentage of hydroxy-aspergillic acid. Yeasts 380588 and 378856 yielded practically no aspergillic acid or hydroxy-aspergillic acid, but did cause the formation of a penicillinlike substance.

#### SUMMARY

A method is described which enhances the production of aspergillic acid by *Aspergillus flavus* in surface cultures. A simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol yielded the highest titers of approximately 0.8 mg per ml in the crude broth. Emphasis is also placed on the importance of assay for aspergillic acid by both the spectrophotometric and the biological methods to verify the production of aspergillic acid, hydroxy-aspergillic acid, or penicillinlike substances.

#### ACKNOWLEDGMENT

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The assistance, also, of Dr. N. F. Coy and Mr. R. Blue of the Squibb Biological Laboratories for their performance of the spectrophotometric and biological assays, respectively, is gratefully acknowledged.

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# CLOSTRIDIUM LACTO-ACETOPHILUM NOV. SPEC. AND THE ROLE OF ACETIC ACID IN THE BUTYRIC ACID FERMENTATION OF LACTATE

J. V. BHAT<sup>1</sup> AND H. A. BARKER

*Division of Plant Nutrition, University of California, Berkeley, California*

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Although the fermentation of lactate by butyric acid bacteria has been studied by a number of eminent bacteriologists (Schattenfroh and Grassberger, 1900; Bredemann, 1909; Boekhout and van Beynum, 1929), including Pasteur (1862) and Beijerinck (1893), it is still a very poorly understood process. A natural butyric acid fermentation of lactate has been reported to occur commonly in low grade silage (van Beynum and Pette, 1936), and, under laboratory conditions, several investigators have easily obtained crude enrichment cultures of butyric acid bacteria using lactate as the main substrate, but nearly all attempts to isolate and propagate these bacteria in pure culture on a lactate medium have failed. In pure culture the bacteria appear to lose their ability to attack lactate, although they can be cultured easily on sugar-containing media. Van Beynum and Pette (1935) finally succeeded in growing these organisms in a lactate medium, but only when an unusually high concentration of yeast autolysate was also provided. Consequently one cannot decide whether the lactate or the yeast autolysate provided the main carbon and energy source. Van Beynum and Pette rightly observe: "There is much uncertainty about the lactate fermentation. As a matter of fact one does not know if it exists as a separate phenomenon, and very little is yet known about the relation between lactate and sugar fermentations."

In the present investigation we have used lactate-decomposing bacteria obtained by the enrichment culture method. By studying their nutritional requirements and chemical activities we have found why butyric acid bacteria cannot be grown in a simple lactate medium and have shown that acetic acid plays an important role in their metabolism.

## EXPERIMENTAL RESULTS

*Enrichment and isolation of lactate-fermenting bacteria.* The investigation was begun by the enrichment and isolation of lactate-fermenting butyric acid bacteria from soil. A medium of the following composition (medium 1) in grams per 100 ml was used: sodium lactate, 1; yeast autolysate, 0.3;  $(\text{NH}_4)_2\text{SO}_4$ , 0.05;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01;  $\text{K}_2\text{HPO}_4$ , 0.05;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002; pH 7, made up with tap water. The medium was inoculated with a small quantity of garden soil and incubated anaerobically at 37 C. Within 36 hours the medium became tur-

<sup>1</sup> The senior author wishes to express his gratitude to the Watumull Foundation for a fellowship which enabled him to work on this problem. Permanent address: Microbiology Department, St. Xavier's College, Bombay, India.

bid, considerable gas was given off, and the pH rose to 8.2 to 8.4. After 72 hours a determination of lactic acid by the method of Friedemann and Graesser (1933) showed that the lactate was completely decomposed.

Several soils of different types were used as inocula for enrichment cultures of this type, and in every instance a vigorous fermentation was obtained within 30 to 48 hours. The predominant bacteria were always medium-sized, actively motile rods, a few of which usually contained oval, central, or subterminal spores. In addition, a few small nonsporulating rods and vibrios were always present when the inoculum was not pasteurized. When a pasteurized soil inoculum was used, a more homogeneous but always less vigorous culture was obtained.

In order to eliminate extraneous bacteria as far as possible before attempting the isolation of pure cultures, one or two successive transfers were made in the same enrichment medium. These cultures always developed within 30 to 40 hours, but were notably less vigorous than the original.

Pure cultures were obtained without special difficulty from the enrichment cultures by the shake culture method of Burri (1902). A solid medium of the same composition as the enrichment medium proved to be satisfactory. Oxygen was removed from the culture tubes by the use of a mixture of pyrogallol and potassium carbonate. Within 20 to 30 hours' incubation at 37 C, the agar in the more heavily inoculated tubes was split by gas, but at the higher dilutions a few well-isolated colonies developed without apparent gas formation. After about 24 hours' incubation at 37 C colonies were visible up to the seventh or eighth dilution, but only at lower dilutions was the agar split by gas.

The colonies of the lactate-decomposing bacteria are generally compact, fluffy, dark gray spheres composed of filamentous outgrowths. They are coarsely lobed and rough-edged; they eventually reach a diameter of 1 to 2 mm. The consistency of the colonies is such that the organisms can be easily drawn into a micro-pipette or transferred with an inoculating needle.

By the repeated use of the shake culture method, nine strains were isolated in pure culture. Each was derived from a different soil.

*Morphological characteristics.* All nine strains are very similar in appearance (figure 1). The average cell dimensions are 0.8 by 4.6 microns, the variation in width being from 0.7 to 0.9 microns and in length from 3 to 8 microns. Spores develop after about 40 hours' incubation in a favorable medium; they are oval in shape and are usually located subterminally, where they cause a distinct bulge in the cell. The average spore size is 1.1 by 1.5 microns. Young vegetative cells are actively motile by means of 20 or more peritrichous flagella. At this stage they are gram-positive. In old cultures most of the cells become gram-negative. Cells generally occur singly or in pairs, though short chains can be seen. When first isolated, three strains appeared to possess small capsules, but on subsequent cultivation in a variety of media, no capsules could be observed in any of the strains.

*Physiological and cultural characteristics.* All nine strains are similar in physiological characteristics. They are obligate anaerobes and their development is favored by the addition of a reducing agent such as sodium thioglycolate to the

medium. The following compounds are readily fermented when supplied in a basal medium containing 3 volumes per cent yeast autolysate and the usual salts: glucose, fructose, galactose, mannose, xylose, arabinose, rhamnose, lactose, sucrose, maltose, trehalose, raffinose, dextrin, glycogen, starch, xylan, mannitol, inositol, inulin, sorbitol, and dulcitol. Glycerol and lactate are attacked very feebly in this medium. However, if 0.8 per cent sodium acetate is also added, both glycerol and lactate are decomposed vigorously. Acid and a moderate amount of gas are formed from the carbohydrates and polyalcohols. The acid is generally a mixture of acetic and butyric acids (see below), and the gas is a mixture of hydrogen and carbon dioxide. Iron milk is slightly acidified without



FIG. 1. *CLOSTRIDIUM LACTO-ACETOPHILUM* STRAIN 3, FORTY-EIGHT-HOUR CULTURE GROWN IN MEDIUM 1 WITH 0.1 PER CENT AGAR  
Free spores are visible in the background.  $\times 1,000$ .

clotting, and a very small amount of gas is formed. Nitrate is not reduced; indole is not formed in a glucose yeast autolysate medium. A little hydrogen sulfide is formed.

Strain 3, which was used for most of the later experimental work, grows at temperatures from 16 to 46 C. The optimum appears to be close to 39 C. The pH range is from 5.6 to 8.4, good growth occurring between pH 6.2 and 7.4. The organisms grow well in a mixed sodium and potassium phosphate buffer in concentrations up to 2 per cent; with 2.4 per cent buffer growth is perceptibly inhibited.

*Classification.* All the strains studied clearly belong to one species which is evidently closely related to *Clostridium butyricum* as defined by Bergey *et al.*

(1939). However, it is definitely stated that the latter species is unable to ferment lactate. Two lactate-fermenting clostridia have been described in the literature, *Clostridium tyrobutyricum* of van Beynum and Pette (1935), and *Granulobacter lactobutyricus* of Beijerinck (1893), but neither of these organisms appears to be able to attack the wide variety of carbohydrates and polyalcohols fermented by our strains. Beijerinck states that *G. lactobutyricus* is unable to attack carbohydrates at all. *C. tyrobutyricum* is described as usually fermenting only glucose, fructose, and lactate. In view of the impossibility of identifying our organism with any previously described species, we have decided to call it *Clostridium lacto-acetophilum*, nov. spec., for reasons which will appear below. Further work may prove that this organism is identical with some other species not now recognized to be able to ferment lactate because the fermentation test was carried out in the absence of acetate (see below). However, in the meantime the name *C. lacto-acetophilum* will serve to identify lactate-fermenting butyric acid bacteria of the type we have described.

*Nutritional requirements.* Shortly after pure cultures of *C. lacto-acetophilum* were first isolated, using a solid lactate yeast autolysate medium (medium 1), the organism was inoculated into a liquid medium of the same composition except for the absence of agar. Growth in this liquid medium was extremely sparse. The addition of 0.1 per cent agar resulted in a slight improvement, and the addition of 2 per cent agar allowed moderately good growth. The failure of the organism to grow satisfactorily in liquid medium 1 could not be due to oxygen inhibition since the addition of 0.05 per cent sodium thioglycolate as a reducing agent did not cause any improvement.

An attempt was made to improve the liquid lactate yeast autolysate medium by adding various substances to it, including larger amounts of yeast autolysate. It soon became evident that growth in the liquid medium is almost proportional to the yeast autolysate concentration up to a level of about 30 volumes per cent. This in itself would not be surprising were it not for the fact that 20 volumes per cent yeast autolysate are required to give as good growth in the liquid medium as can be obtained with only 3 volumes per cent in an otherwise identical solid medium.

Previous experiments conducted in this laboratory (Barker, 1947; Bornstein and Barker) with another bacterium, *Clostridium kluyveri*, had shown that an abnormally high requirement for yeast autolysate may be due to a need for acetic acid, which is always present in yeast autolysate in small amounts. We therefore tried adding 0.3 per cent sodium acetate to the liquid lactate medium containing 3 volumes per cent yeast autolysate. The results were very striking; excellent growth occurred in the presence of acetate, whereas in its absence growth was extremely poor. Quantitative experiments on the relation between acetate concentration and growth measured with an Evelyn colorimeter showed that the growth rate and the maximum cell yield increase with sodium acetate concentration up to about 0.8 per cent. Table 1 shows that the total amount of lactate decomposed also increases with the initial acetate concentration.

Several other substances were tested to determine whether they can substitute



for acetate in stimulating growth and lactate decomposition. The substances so tested were formate, propionate, butyrate, fumarate, succinate, malate, tartrate, citrate, pyruvate, ethanol, and glucose. They were used at concentrations of 0.1 and 0.5 per cent. Both lactate and lactate-acetate media were used as controls. It was found that only pyruvate can substitute for acetate in favoring both growth and lactate decomposition. Glucose, either alone or in combination with lactate, supports very good growth, but it does not accelerate the disappearance of lactate.

It should be noted that *C. lacto-acetophilum* differs markedly from *C. kluyveri* with respect to the substrates that can be substituted for acetate. The latter organism can use propionate, butyrate, and to a lesser extent valerate, but cannot use pyruvate.

TABLE 1

*The dependence of growth and lactate decomposition on the acetate supply*

INITIAL ACETATE	LACTATE DECOMPOSED	RELATIVE GROWTH
<i>mm/100 ml</i>	<i>mm/100 ml</i>	
0.05	0.44	177
0.79	1.89	446
1.52	2.89	680
2.26	3.78	809
3.00	4.67	888
3.73	5.22	982
4.47	5.78	1,107
5.94	6.22	1,177
7.40	6.55	1,192

Medium: sodium lactate, 8.8 mm per 100 ml, yeast autolysate, 3 volumes per cent, the salts of medium 1, and the indicated sodium acetate concentrations. All cultures were incubated 3 days at 37 C under anaerobic conditions. Strain 3.

When 0.6 to 0.8 per cent acetate is added to the culture medium, the concentration of yeast autolysate needed to give maximal growth is greatly reduced. About 3 volumes per cent is quite adequate, whereas at lower concentrations the cell yield decreases. However, the yeast autolysate level can be still further reduced to about 0.1 volume per cent without limiting growth if the medium is supplemented with 0.01  $\mu$ g biotin and 10  $\mu$ g *para*-aminobenzoic acid per 100 ml. A few attempts to replace yeast autolysate completely by known growth factors and amino acid mixtures were unsuccessful. In a medium containing lactate, acetate, biotin, *para*-aminobenzoic acid, and 0.01 volume per cent yeast autolysate, the organism failed to respond favorably to any of the following compounds or preparations: thiamine, nicotinic acid, riboflavin, pyridoxine, folic acid, acid-hydrolyzed casein, peptone, and tryptone.

The following medium (medium 2), which supports excellent growth of *C. lacto-acetophilum*, was developed on the basis of the foregoing experiments: sodium lactate, 1 g; sodium acetate, 0.8 g; yeast autolysate, 0.5 ml (0.05 g dry

weight); sodium thioglycolate, 0.05 g;  $(\text{NH}_4)_2\text{SO}_4$ , 0.05 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g;  $\text{K}_2\text{HPO}_4$ , 0.05 g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 g; biotin, 0.01  $\mu\text{g}$ ; *para*-aminobenzoic acid, 10  $\mu\text{g}$ ; distilled water, 100 ml; pH 7. Heavier growth is obtained if lactate is replaced by glucose.

*Fermentation products.* The observation that the amount of lactate decomposed is dependent upon the initial acetate concentration suggests that acetate is consumed in the lactate-acetate fermentation. This possibility was verified experimentally, and the products of the lactate-acetate fermentation in a growing culture were determined quantitatively by methods previously described (Barker and Haas, 1944). The main products are butyric acid, carbon dioxide, and hydrogen (table 2, column 3).

The influence of acetate concentration on acetate utilization and butyrate formation is shown in table 3. Only at the lowest initial concentration is there a

TABLE 2

*Fermentation products*

COMPOUND	ENRICHMENT CULTURE	PURE CULTURE, STRAIN 3		
	Substrates			
	Lactate, 1%	Lactate, 1% + acetate, 0.8%	Pyruvate, 1%	Glucose, 1%
Acetic acid.....	50	-32	33	28
Butyric acid.....	35	65	33	73
Carbon dioxide.....	55.5	100	93	190
Hydrogen.....	10	59	30	182
Carbon recovery (%)....	98	99	97	90
Redox index.....	1.39	1.05	0.96	1.12

The figures represent yields in moles per 100 moles of fermented substrate. Each medium contained the salts of medium 1, 3 volume per cent yeast autolysate, and the indicated substrate concentrations. Cultures were incubated at 37 C until fermentation ceased.

net production of both acetate and butyrate. At higher acetate concentrations there is always a disappearance of acetate, which increases with concentration. It should be noted that added acetate is never entirely used up. The final concentration is always above 1 millimole per 100 ml. In this respect acetate behaves quite differently from most other substrates, like glucose and lactate, which under favorable conditions are completely fermented by this organism. The explanation for this anomalous behavior appears to be that the utilization of acetate depends upon the concentration of butyrate. Column 5, table 3, shows that the butyrate-acetate concentration ratio in the fermented medium never exceeds a value of about 2.6 and is remarkably constant and independent of the initial acetate concentration over a wide range. This indicates the existence of some sort of equilibrium between acetate and butyrate. The ratio of butyrate formed to acetate used (column 6, table 3) is more variable than the final concentration

ratio. The former ratio is infinite at the lowest acetate level, indicating that all the butyrate is derived from lactate. At intermediate acetate levels, approximately one mole of acetate is used for each mole of butyrate produced. At higher levels, the molar quantity of acetate used considerably exceeds the butyrate formed. Since it is theoretically impossible to use more than one mole of acetate plus one mole of lactate in the formation of one mole of butyrate, this result must mean that some other product, such as acetone, is being formed under these conditions.

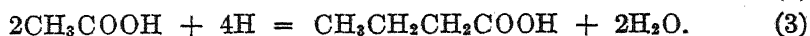
The lactate-acetate fermentation is evidently analogous to the ethanol-acetate fermentation of *C. kluyveri*. In the latter process, the ethanol is oxidized to an active form of acetic acid, which condenses with the substrate acetate to give an intermediate compound that is ultimately reduced to butyric acid (Barker,

TABLE 3  
*Influence of acetate concentration on the lactate acetate fermentation*

mm/100 ML				FINAL BUTYRATE FINAL ACETATE	BUTYRATE FORMED ACETATE USED
Acetate			Butyrate formed		
Added	Final	Used			
0.05	0.41	-0.36	0.295	0.72	∞
1.27	1.04	0.23	1.24	1.19	5.39
2.49	1.01	1.48	2.23	2.21	1.51
3.76	1.13	2.58	2.95	2.62	1.02
4.93	1.50	3.43	3.90	2.60	1.14
6.15	1.82	4.32	4.74	2.60	1.10
7.37	2.03	5.34	5.27	2.60	0.99
8.59	2.14	6.45	5.55	2.60	0.86
9.81	2.25	7.56	5.85	2.60	0.77
11.03	3.93	7.10	4.72	1.61	0.66
12.25	5.20	7.05	3.47	0.67	0.49

Medium no. 1 containing 3 volumes per cent yeast autolysate, 1 per cent sodium lactate, and the specified amount of sodium acetate was used. The cultures were incubated for 5 days at 37 C until fermentation had ceased. Strain 3.

Kamen, and Bornstein, 1945). In the lactate-acetate fermentation of *C. lacto-acetophilum* lactate is evidently oxidized to carbon dioxide and acetic acid or a derivative thereof. It will be noted that one mole of carbon dioxide is formed per mole of lactate fermented as is required of such a mechanism. As in the *C. kluyveri* fermentation, butyric acid is probably formed by a condensation of two moles of acetic acid or related compound, followed by a reduction. The postulated fermentation mechanism may be schematically represented as follows:



The need for an outside supply of acetate in the decomposition of lactate may be explained by considering the oxidation-reduction relations involved in the fer-

mentation. These relations are independent of any specific mechanism but for convenience we shall assume the reactions postulated above. The oxidation of 100 moles of lactate to acetate and carbon dioxide (equation 1) releases 400 equivalents of hydrogen, which must be used in reduction processes if the fermentation is to maintain itself. Part of the hydrogen (118 equivalents) appears as hydrogen gas (equation 2). The remainder can only be used in the formation of butyric acid or the reduction of small quantities of hydrogen acceptors present in the yeast autolysate. The latter will be quantitatively important only when the concentration of acetate is low. The reduction of 100 moles of acetate (derived from the oxidation of 100 moles of lactate) to 50 moles of butyrate would consume only 200 equivalents of hydrogen, leaving an excess of 82 equivalents. Extra acetate is required to form a sufficient quantity of a hydrogen acceptor to react with this excess. These relations may be summarized by saying that the fermentation of lactate by this organism cannot proceed without the intervention of an outside oxidizing agent, which in this case is acetic acid. In this connection it should be noted that glycerol also cannot be decomposed by this organism unless acetate is added to act as an oxidizing agent.

Pyruvate and glucose, in contrast to lactate and glycerol, can be fermented in the absence of added acetate (table 2, columns 4, 5). The nonnecessity of extra acetate as an oxidant in the pyruvate fermentation is easily explained. Pyruvate is in a more oxidized state than lactate; when it is converted to acetate and carbon dioxide, two equivalents of hydrogen become available. Since this is just enough hydrogen to make possible the reduction of the acetate simultaneously formed, no outside source of acetate is necessary. In fact, there is a considerable net accumulation of acetate in the fermentation of pyruvate due to the fact that part of the available hydrogen is not used for butyrate formation but is given off as hydrogen gas.

The decomposition of glucose in the absence of added acetate must be explained on a different basis since glucose, unlike pyruvate, is in the same oxidation state as lactate. The fermentation of glucose alone is possible because of the relatively much larger evolution of gaseous hydrogen. In the pyruvate fermentation, involving a two-step oxidation to acetate and carbon dioxide, the yield of hydrogen gas is 30 mm per 100 mm of substrate, and in the lactate fermentation, involving a four-step oxidation, the yield is 59 mm hydrogen, a proportional amount. On the same basis one would expect that the glucose fermentation, which involves eight oxidation steps, would yield about 120 mm hydrogen. However, the observed hydrogen production is 182 mm, about 50 per cent greater than this expectation. From these results one may conclude that some intermediate hydrogen donor in the glucose fermentation, possibly triosephosphate, contributes more heavily to gaseous hydrogen formation than do the compounds involved in the lactate and pyruvate fermentations.

*Fermentation of lactate by enrichment cultures.* Although added acetate is essential for the fermentation of lactate by pure cultures of *C. lacto-acetophilum*, this is not true of the first enrichment cultures for this organism. It has been repeatedly observed that a liquid lactate yeast autolysate medium inoculated with

soil supports an excellent growth of anaerobic bacteria, most of which appear to be *C. lacto-acetophilum*, and the lactate is rather rapidly and completely decomposed. When a transfer is made from the first enrichment culture to the same medium (without acetate), the growth is somewhat poorer and the lactate decomposition is less complete. For example, in one experiment only 82 per cent of the lactate was used up in such a culture, whereas after a second transfer only 26 per cent of the lactate was consumed. These results show that some factor, possibly a second organism, is operating in the first enrichment cultures in such a way as to make the addition of acetate unnecessary. By making successive transfers, this factor is lost and acetate becomes essential just as it is in pure culture experiments.

In order to find what oxidant is substituted for acetate in lactate enrichment cultures, the fermentation products were determined quantitatively (table 2, column 2). The analytical results evidently contain some errors, as evidenced by the high redox index, but they nevertheless are satisfactory to show several striking differences between enrichment and pure culture fermentations. Acetic acid is produced rather than consumed in the enrichment culture, and the yields of butyrate, carbon dioxide, and hydrogen are all greatly reduced. The low yield of carbon dioxide, coupled with the high yield of acetic acid, clearly indicates the utilization of carbon dioxide as an important oxidizing agent in the enrichment culture. The fermentation is very similar to that caused by *Butyribacterium rettgeri* (Barker, Kamen, and Haas, 1945), which has been proved to involve a reduction of carbon dioxide to acetic acid. However, we have not been able to obtain any evidence for the occurrence of species of *Butyribacterium* in the enrichment cultures. The organism or factor responsible for the anomalous behavior of enrichment cultures remains to be discovered.

#### DISCUSSION

We have shown that a lactate-fermenting butyric acid bacterium, *Clostridium lacto-acetophilum*, can be readily isolated from soil and that the ability of this organism to decompose lactate in pure culture is dependent upon the presence of acetate. The catabolism of this organism may be described as an oxidation of lactate to acetate and carbon dioxide, coupled with a reductive condensation of acetate to butyrate. A little gaseous hydrogen is also formed. Since the amount of acetate produced from lactate is insufficient to react with all the hydrogen derived from lactate, extra acetate must be added to maintain the reaction.

*C. lacto-acetophilum* is not the only organism requiring acetate as a primary oxidant. A similar type of fermentation is catalyzed by *C. kluyveri*, which oxidizes ethanol to acetic acid and then converts the acetic acid first to butyric acid and later to caproic acid (Barker, Kamen, and Bornstein, 1945). The catabolic processes of the two organisms differ mainly with respect to the substrates that can be oxidized and the products of acetate condensation and reduction. Experiments with isotope-labeled acetic acid (Wood *et al.*, 1944, 1945; Barker, Kamen, and Haas, 1945) clearly show that other butyric acid bacteria,

like *C. butylicum*, *C. acetobutylicum*, and *Butyribacterium rettgeri*, also use acetate as a primary oxidant, even though they do not need to have it supplied from an outside source. In such organisms, accessory oxidants, such as carbon dioxide, butyric acid, or acetone, are available to supplement acetic acid. In addition gaseous hydrogen may be formed.

Our results explain why previous investigators have had so little success in growing butyric acid bacteria on lactate media in pure culture. Their bacteria did not lose the ability to ferment lactate, as has commonly been supposed; they simply were unable to oxidize lactate in the absence of acetate. When acetate was unintentionally added, as was done by van Beynum and Pette (1935) through the use of large amounts of yeast extract, the bacteria grew and decomposed lactate. It seems likely that the ability to ferment lactate and glycerol is much more widely distributed among butyric acid bacteria than has been previously reported. At least some species that are now thought to be unable to attack lactate and glycerol will probably be found to do so when acetate is added to the test media. The addition of 0.5 per cent sodium acetate to all media for butyric acid bacteria will probably be found advantageous.

#### SUMMARY

The isolation and characteristics of a lactate-fermenting butyric acid bacterium, *Clostridium lacto-acetophilum*, nov. spec., are described. It is shown that the decomposition of lactate by this organism is dependent on the presence and simultaneous utilization of acetate. The role of acetate in butyric acid fermentations is discussed.

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# ANTIBIOTIC PRODUCTION BY MARINE MICROORGANISMS<sup>1</sup>

WILLIAM D. ROSENFELD<sup>2</sup> AND CLAUDE E. ZOBELL

*Scripps Institution of Oceanography, University of California, La Jolla, California*

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The multiplication of most nonmarine bacteria is inhibited by sea water (Kofínek, 1927). ZoBell (1941) found that only from 4 to 15 per cent as many bacteria from soil, sewage, and other fresh-water or terrestrial sources formed colonies on nutrient agar prepared with sea water as on a similar medium prepared with distilled water. Sea water is also bactericidal for many nonmarine bacteria, gram-positive organisms being more sensitive than gram-negative forms (Beard and Meadowcroft, 1935; ZoBell, 1936). This was demonstrated by suspending pure or mixed cultures of bacteria from various fresh-water sources in sea water or other mineral solutions and determining the percentage of survival after different periods of time. Although gram-negative organisms display resistance to the lethal action of sea water, their viability in this medium varies widely among different species. In an investigation employing enteric bacteria, Trawinski (1929) reported survival periods in sea water ranging from 12 hours for *Shigella dysenteriae* to 23 days for *Salmonella enteritidis*. Carpenter *et al.* (1938) noted that natural sea water killed 80 per cent of the organisms in sewage within half an hour.

The bacteriostatic and bactericidal effects of sea water are greater than can be accounted for upon a basis of its salinity or osmotic pressure. Not only is natural sea water more bactericidal than synthetic sea water (ZoBell, 1946; ZoBell and Feltham, 1933), but it is also more bactericidal than heat-treated sea water (Kiribayashi and Aida, 1933; Waksman and Hotchkiss, 1937; ZoBell, 1936). De Giaksa (1889) observed that pathogens rapidly perish in raw sea water, although they may survive almost indefinitely in heat-treated sea water. Water from the Black Sea was found by Krassilnikov (1938) to be germicidal for terrestrial bacteria until it was boiled. He confirmed the observations of Beard and Meadowcroft (1935) and ZoBell (1936) that the bactericidal potency of sea water was decreased but not destroyed by passing it through Berkefeld, Chamberland, Coors, or similar filters.

Lacking the properties of bacteriophage, the bactericidal property of sea water is attributed primarily to its content of antibiotic substances produced by microorganisms. Credence is lent to this view by the observation that the bactericidal principle occurs in greatest concentration in samples of sea water recently collected from zones of maximum bacterial population. The experimental results reported below demonstrate the production of antibiotic substances by several species of microorganisms native to the sea.

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<sup>2</sup> Present address: California Research Corporation, La Habra, California.

## EXPERIMENTAL RESULTS

Fifty-eight pure cultures of marine microorganisms were tested for their antimicrobial activities. The cultures employed as potential antagonists were taken from a collection previously described by ZoBell and Upham (1944) and were maintained in a medium of the following composition:

Difco peptone .....	5.0 g
Difco yeast extract .....	1.0 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	1.0 g
FeSO <sub>4</sub> · (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> · 6H <sub>2</sub> O .....	0.1 g
Aged sea water (75 per cent) .....	1,000 ml

The solution was adjusted to pH 7.4 to 7.6 before autoclaving. When a solid medium was required, 2.0 per cent of agar was added to this solution prior to sterilization.

TABLE 1  
*Antimicrobial behavior of marine microorganisms*

ANTAGONISTIC GENUS	SPECIES TESTED	SPECIES INHIBITING FRESH-WATER OR TERRESTRIAL ORGANISMS
<i>Bacillus</i> .....	9	4
<i>Micrococcus</i> .....	6	3
<i>Actinomyces</i> .....	2	1
<i>Serratia</i> .....	1	1
<i>Pseudomonas</i> .....	19	0
<i>Vibrio</i> .....	11	0
<i>Flavobacterium</i> .....	5	0
<i>Achromobacter</i> .....	4	0
<i>Sarcina</i> .....	1	0
Totals .....	58	9

All fresh-water or terrestrial species were tested to ensure their growth in this sea-water medium before subjecting them to the effects of marine antagonists. Both antagonistic and test inocula were taken from cultures grown for 48 hours at 27 C.

Antimicrobial effects were surveyed in pour plates, each containing 20 ml of nutrient agar and seeded with 0.2 ml of a test culture. After the medium had solidified, the marine species under investigation were streaked over quadrants of the plates, which were then incubated at 27 C. Plates were examined at frequent intervals over a period of 17 days for growth suppression of the test organisms, as evidenced by clearing zones adjacent to the lines of growth of marine antagonists. Table 1 outlines the results noted and summarizes the distribution of antagonistic species among the various genera investigated.

Although two antagonistic organisms were gram-negative, the majority of species exhibiting inhibitory powers were members of the gram-positive genera *Bacillus* and *Micrococcus*.

The susceptibility of the test organisms is described in tables 2, 3, and 4. Of the 11 gram-positive cultures employed, only *Staphylococcus aureus* and *Strepto-*

TABLE 2  
*Antimicrobial spectrum of marine Bacillus species*

TEST SPECIES	INHIBITED BY			
	<i>B. borborokoites</i>	<i>B. abyssus</i>	<i>B. thalassokoites</i>	<i>B. submarinus</i>
<i>Bacillus anthracis</i> .....	+	+	—	—
<i>B. megatherium</i> .....	—	—	—	—
<i>B. mycoides</i> .....	+	+	+	+
<i>B. subtilis</i> .....	—	—	—	—
<i>Corynebacterium pseudodiphthericum</i> ..	+	—	—	—
<i>Micrococcus roseus</i> .....	+	+	+	+
<i>Mycobacterium lacticola</i> .....	—	—	—	—
<i>Proteus vulgaris</i> .....	—	—	—	—
<i>Salmonella typhimurium</i> .....	—	—	—	—
<i>Sarcina lutea</i> .....	+	+	+	+
<i>Shigella paradysenteriae</i> .....	—	—	—	—
<i>Staphylococcus aureus</i> .....	—	—	—	—
<i>S. citreus</i> .....	+	+	+	+
<i>Streptococcus faecalis</i> .....	—	—	—	—

TABLE 3  
*Antimicrobial spectrum of marine Micrococcus species*

TEST SPECIES	INHIBITED BY		
	<i>M. maripuniceus</i>	<i>M. sedimentus</i>	<i>M. infimus</i>
<i>Bacillus anthracis</i> .....	+	—	*
<i>B. megatherium</i> .....	—	—	*
<i>B. mycoides</i> .....	—	—	—
<i>B. subtilis</i> .....	—	—	—
<i>Corynebacterium pseudodiphthericum</i> ..	—	—	*
<i>Micrococcus roseus</i> .....	—	—	—
<i>Mycobacterium lacticola</i> .....	+	—	*
<i>Proteus vulgaris</i> .....	—	—	—
<i>Salmonella typhimurium</i> .....	—	—	—
<i>Sarcina lutea</i> .....	+	+	+
<i>Shigella paradysenteriae</i> .....	—	—	*
<i>Staphylococcus aureus</i> .....	—	—	—
<i>S. citreus</i> .....	+	—	*
<i>Streptococcus faecalis</i> .....	—	—	*

\* This species not tested.

*coccus faecalis* failed to undergo inhibition by one or more marine species. Three gram-negative species were tested, and none of these was susceptible.

An attempt was made to demonstrate the presence of the inhibitory principle in cell-free solutions prepared by filtration of the antagonistic cultures. The

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$\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ . . . . .	0.1 g
Aged sea water (75 per cent) . . . . .	1,000 ml

The solution was adjusted to pH 7.4 to 7.6 before autoclaving. When a solid medium was required, 2.0 per cent of agar was added to this solution prior to sterilization.

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<i>Serratia</i> . . . . .	1	1
<i>Pseudomonas</i> . . . . .	19	0
<i>Vibrio</i> . . . . .	11	0
<i>Flavobacterium</i> . . . . .	5	0
<i>Achromobacter</i> . . . . .	4	0
<i>Sarcina</i> . . . . .	1	0
Totals . . . . .	58	9

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Antimicrobial effects were surveyed in pour plates, each containing 20 ml of nutrient agar and seeded with 0.2 ml of a test culture. After the medium had solidified, the marine species under investigation were streaked over quadrants of the plates, which were then incubated at 27 C. Plates were examined at frequent intervals over a period of 17 days for growth suppression of the test organisms, as evidenced by clearing zones adjacent to the lines of growth of marine antagonists. Table 1 outlines the results noted and summarizes the distribution of antagonistic species among the various genera investigated.

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<i>Bacillus anthracis</i> .....	+	+	—	—
<i>B. megatherium</i> .....	—	—	—	—
<i>B. mycoides</i> .....	+	+	+	+
<i>B. subtilis</i> .....	—	—	—	—
<i>Corynebacterium pseudodiphthericum</i> ..	+	—	—	—
<i>Micrococcus roseus</i> .....	+	+	+	+
<i>Mycobacterium lacticola</i> .....	—	—	—	—
<i>Proteus vulgaris</i> .....	—	—	—	—
<i>Salmonella typhimurium</i> .....	—	—	—	—
<i>Sarcina lutea</i> .....	+	+	+	+
<i>Shigella paradysenteriae</i> .....	—	—	—	—
<i>Staphylococcus aureus</i> .....	—	—	—	—
<i>S. citreus</i> .....	+	+	+	+
<i>Streptococcus faecalis</i> .....	—	—	—	—

TABLE 3  
*Antimicrobial spectrum of marine Micrococcus species*

TEST SPECIES	INHIBITED BY		
	<i>M. maripunicus</i>	<i>M. sedimentus</i>	<i>M. infimus</i>
<i>Bacillus anthracis</i> .....	+	—	*
<i>B. megatherium</i> .....	—	—	*
<i>B. mycoides</i> .....	—	—	—
<i>B. subtilis</i> .....	—	—	—
<i>Corynebacterium pseudodiphthericum</i> ...	—	—	*
<i>Micrococcus roseus</i> .....	—	—	—
<i>Mycobacterium lacticola</i> .....	+	—	*
<i>Proteus vulgaris</i> .....	—	—	—
<i>Salmonella typhimurium</i> .....	—	—	—
<i>Sarcina lutea</i> .....	+	+	+
<i>Shigella paradysenteriae</i> .....	—	—	*
<i>Staphylococcus aureus</i> .....	—	—	—
<i>S. citreus</i> .....	+	—	*
<i>Streptococcus faecalis</i> .....	—	—	*

\* This species not tested.

*coccus faecalis* failed to undergo inhibition by one or more marine species. Three gram-negative species were tested, and none of these was susceptible.

An attempt was made to demonstrate the presence of the inhibitory principle in cell-free solutions prepared by filtration of the antagonistic cultures. The

inhibitory organisms were grown in the broth described above for periods of 9 to 11 days, after which they were passed through Seitz or Mandler filters. The reaction of all filtrates approximated pH 8 and in each case was adjusted to pH 7.2 before employment of the sterile solutions as inhibitory agents. Pour plates of susceptible organisms were prepared in the manner already outlined. Sterile discs of filter paper were saturated with the culture filtrates and applied to the

TABLE 4  
*Antimicrobial spectrum of marine microorganisms*

TEST SPECIES	INHIBITED BY	
	<i>Actinomyces marinolimosus</i>	<i>Serratia marinorubra</i>
<i>Bacillus anthracis</i> .....	—	+
<i>B. megatherium</i> .....	+	+
<i>B. mycoides</i> .....	+	—
<i>B. subtilis</i> .....	—	+
<i>Corynebacterium pseudodiphthericum</i> .....	—	—
<i>Micrococcus roseus</i> .....	+	—
<i>Mycobacterium lacticola</i> .....	—	—
<i>Proteus vulgaris</i> .....	—	—
<i>Salmonella typhimurium</i> .....	—	—
<i>Sarcina lutea</i> .....	+	—
<i>Shigella paradysenteriae</i> .....	—	—
<i>Staphylococcus aureus</i> .....	—	—
<i>S. citreus</i> .....	—	—
<i>Streptococcus faecalis</i> .....	—	—

TABLE 5  
*Decreases in antimicrobial activity resulting from filtration of antagonistic cultures*

ANTAGONIST		SUSCEPTIBLE SPECIES	
Species	Filter	Tested	Inhibited
<i>Actinomyces marinolimosus</i>	Seitz	4	1
<i>Bacillus borborokoites</i>	Mandler	6	1
<i>B. abyseus</i>	Seitz	5	1
<i>B. thalassokoites</i>	Seitz & Mandler	4	0
<i>B. submarinus</i>	Seitz	4	1
<i>Micrococcus maripuniceus</i>	Seitz	4	0
<i>M. sedimeneus</i>	Mandler	1	0
<i>Serratia marinorubra</i>	Mandler	3	0

agar surface. Such plates were incubated for 6 days, during which they were examined frequently for the presence of inhibition zones. The results are given in table 5.

Little antagonistic activity was displayed by the cell-free preparations. Although such results may suggest that the inhibitory substance is intimately associated with its parent cell, there is the possibility that the active principle was removed by adsorption on the filter.

## DISCUSSION

Quantitative evidence of the effect of marine microorganisms upon the inhibitory property of sea water has not been obtained. However, a presumptive interrelationship may be inferred from the incidence of antagonistic species. The behavior of antagonistic cultures when passed through germ-proof filters resembles that of sea water, the two undergoing similar decreases in antimicrobial potency. Further evidence for this correlation of activity is observed in a comparison of data reported herein with that testing the effect of sea water upon nonmarine bacteria. Of six organisms shown by Krassilnikov (1938) to be inhibited by unheated sea water and common to both investigations, only *Staphylococcus aureus* failed to demonstrate a bacteriostatic response to marine bacteria. It is also significant that the response of test species to inhibition by marine organisms appears to parallel the gram reaction. This is in accordance with the general observation that gram-positive bacteria are more often inhibited by sea water than are gram-negative species.

Isolations of specific antibiotics produced by marine bacteria have not been attempted, but it is evident that various species of microorganisms indigenous to the sea elaborate antimicrobial substances. The survey reported here, although very limited in scope, suggests that the marine environment should be considered as a potential source of antibiotics.

## SUMMARY

Of 58 species of marine microorganisms tested, 9 have demonstrated antibiotic activity against nonmarine forms. The most actively antagonistic marine genera were *Bacillus* and *Micrococcus*.

Similarities in the behavior of sea water and of antagonistic marine cultures indicate that the bacteriostatic or bactericidal activity of the former may be at least partially due to an autochthonous flora of antibiotic-producing organisms.

It is suggested that the sea may represent a reservoir of microbial antagonists of possible importance.

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## NOTES

### STREPTOMYCIN TOLERANCE OF SAPROPHYTIC AND PATHOGENIC FUNGI

M. L. LITTMAN

*Department of Pathology and Bacteriology, Tulane University School of  
Medicine, New Orleans, Louisiana*

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Relatively little information is available concerning the effect of streptomycin on the growth of pathogenic fungi. During the course of an investigation of fungal culture media (reported elsewhere) it became necessary to test the growth of a number of saprophytic and pathogenic fungi on agar containing 30 units of streptomycin sulfate per ml (Winthrop, Cutter). It was observed that growth of the following fungi was found to be unaffected by the concentration of antibiotic employed: *Blastomyces dermatitidis* (2 strains), *Blastomyces brasiliensis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporotrichum schenckii*, *Hormodendrum pedrosoi*, *Hormodendrum compactum*, *Phialophora verrucosa*, *Cryptococcus neoformans*, *Candida albicans*, *Candida candida*, *Microsporium audouinii*, *Microsporium canis*, *Microsporium gypseum*, *Trichophyton schoenleinii*, *Trichophyton violaceum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Monosporium apiospermum*, *Geotrichum* sp., *Penicillium expansum*, *Aspergillus herbariorum*, *Rhizopus nigricans*, *Neurospora sitophila*, *Fusarium*, *Alternaria*, *Cladosporium*, *Mucor mucedo*.

Although the fungi listed above may possibly be inhibited by stronger concentrations of streptomycin, their tolerance to 30 units per ml *in vitro* indicates that systemic and cutaneous infections caused by the pathogenic species are not likely to respond well to clinical treatment with streptomycin.



# NUTRITIONAL STUDIES ON *PIRICULARIA ORYZAE*<sup>1</sup>

F. W. LEAVER, J. LEAL,<sup>2</sup> AND C. R. BREWER<sup>3</sup>

*Camp Detrick, Frederick, Maryland*

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*Piricularia oryzae* Cav., the cause of the disease known as "rice blast" which is commonly found in many of the rice-growing areas of the world, varies considerably in quantity and quality of growth on natural media, as shown by Henry and Andersen (1944). This paper is a report of the development of chemically defined ("synthetic") media for *P. oryzae* with the aim of producing growth and sporulation comparable to the best natural media and of reducing the degree of variation in conidia production on subculture below that found when the fungus is grown on a natural medium such as rice polish agar.

Little is known of the nutritional requirements of *P. oryzae*. Tochinal and Nakano (1940) reported growth on a synthetic medium containing only  $\text{NH}_4\text{NO}_3$ ,  $\text{MgSO}_4$ , xanthine, glucose, and inorganic salts. Attempts in this laboratory to confirm their work were unsuccessful.

## METHODS

A simplified medium (chemically defined except for the presence of purified agar and acid-hydrolyzed "vitamin-free" casein) was prepared for the cultivation of the fungus. All ingredients were included which are commonly required by fungi, and several compounds were added which had shown evidence of being beneficial in preliminary experiments (table 1). The usual precautions as to cleanliness of glassware and purity of reagents necessary in nutritional studies were observed.

The vitamin-free casein was hydrolyzed with  $\text{H}_2\text{SO}_4$ , which was subsequently nearly neutralized with  $\text{Ba}(\text{OH})_2$  to remove all but a small amount of sulfate. After the precipitate was washed with distilled water, the hydrolyzed casein solution was clarified with charcoal at pH 3.5 to 4.0 until it was nearly colorless. The concentrations of hydrolyzed casein reported in the tables were computed on the basis of the amino nitrogen content of the hydrolyzates. A bacteriological assay<sup>4</sup> of the medium (table 1) with *Lactobacillus casei* showed that it contained no biotin, no pantothenic acid, and approximately 0.15  $\mu\text{g}$  per ml of nicotinic acid. A chemical assay<sup>4</sup> showed less than 0.04  $\mu\text{g}$  per ml of thiamine.

Commercial bacteriological agar was washed three times with a mixture of equal parts of pyridine and ethyl alcohol, then with distilled water until no trace

<sup>1</sup> Studies conducted at Camp Detrick, Frederick, Maryland, from August, 1944, to November, 1946.

<sup>2</sup> 1st Lt., AUS; T/5, AUS; respectively.

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<sup>4</sup> Analysis by Capt. D. H. Bornor, AUS.

of pyridine could be detected by odor, and finally washed three more times with distilled water. It was dried at 50 C for a minimum of 3 days. All of the growth media reported in this paper were solidified with 2.0 per cent of this purified agar.

All cultures were grown in selected 18-by-150-mm pyrex culture tubes in which the media were slanted uniformly. The average area was found to be 9.6 cm<sup>2</sup>, with variations of not more than 0.5 cm<sup>2</sup> among individual tubes.

The stock cultures of *P. oryzae*<sup>5</sup> were grown on 2 per cent rice polish, 2 per cent agar slants. Conidia from several 5- to 6-day-old cultures were suspended in sterile distilled water by violent shaking. The suspensions were combined and used for inoculating the media in each experiment. This method produced a low concentration of conidia, but the suspension was relatively free from mycelial fragments and undesirable material from the rice polish agar slant. Ten to 20 thousand conidia suspended in 0.5 ml of sterile distilled water were added to each slant by means of a syringe. The suspension was distributed over the entire surface of the slant by tilting and the excess liquid absorbed on the cotton plug. Repeated tests showed that for the size of the cotton plug used, the amount of excess liquid absorbed was so small that no contamination occurred because of the wetting of the plug. Uniform growth occurred over the agar surface, and the variation between replicate tubes was held to a minimum.

Four replicates of each medium were inoculated and incubated in the dark for 5 days. In the early experiments, incubation was at 22 to 28 C; in later experiments incubation was at 25 to 27 C. The amount of growth obtained was estimated visually. The degree of sporulation was determined microscopically by counting in a Howard chamber the sample obtained by suspending the conidia in each tube in 10 ml water. The conidia counts given in this paper are averages of four replicate tubes. All results on vitamin requirements were analyzed statistically.

## RESULTS

*Vitamin requirements.* The requirements for the B vitamins were determined by adding the following vitamins of the B complex to the basal medium (table 1): thiamine hydrochloride, calcium pantothenate, nicotinic acid, biotin, *d*-riboflavin, and pyridoxine. To test the effects of these vitamins, they were omitted singly and in groups from a medium containing all of these six vitamins. The omission of nicotinic acid, riboflavin, pyridoxine, and calcium pantothenate had no significant effect on the yield of conidia; however, when either thiamine or biotin was omitted no growth occurred. The results are summarized in table 1. Other experiments showed that neither *p*-aminobenzoic acid nor a folic acid concentrate<sup>6</sup> affected the growth or production of conidia of *P. oryzae*.

Six levels of biotin were added to a medium containing only chemically known ingredients other than agar to determine the optimal biotin level for growth and

<sup>5</sup> The culture of *P. oryzae* was obtained from Dr. E. C. Tullis, U. S. D. A., Beaumont, Texas.

<sup>6</sup> We wish to thank Dr. R. J. Williams, University of Texas, for the gift of this folic acid concentrate.

production of conidia. The results (table 2) indicate that the optimal level lies between 0.001 and 0.01  $\mu\text{g}$  per ml. This experiment confirmed, in a more highly purified medium, the previous findings that no B complex vitamins other than thiamine and biotin are required.

An attempt was made to replace biotin with cysteine and pimelic acid, as Eakin and Eakin (1942) have done with *Aspergillus niger*. *P. oryzae* did not grow on the basal medium when either cysteine (50  $\mu\text{g}$  per L) or pimelic acid (65  $\mu\text{g}$  per L) or a combination of both was added in place of biotin.

TABLE 1

*Effect of B complex vitamins on growth and production of conidia by P. oryzae*

VITAMIN OMITTED FROM MIXTURE*	GROWTH	SPORES IN THOUSANDS/CM <sup>2</sup>		
		Subculture no.		
		1	2	3
None.....	good	16.7		
Nicotinic acid.....	good	10.3		
Ca-pantothenate.....	good	11.1		
d-Riboflavin.....	good	14.0		
Thiamine-HCl.....	none	0.0		
Pyridoxine.....	good	12.2		
Biotin.....	none	0.0		
None.....	good	108	430	81
Nicotinic acid	good	102	670	203
Ca-pantothenate				
d-Riboflavin				
Pyridoxine				
Rice polish agar control.....	good	62	81	59

Medium (g/L): sucrose, 5.0; acid-hydrolyzed "vitamin-free" casein, 1.0; agar, 20;  $\text{K}_2\text{HPO}_4$ , 0.5; glycerol, 0.05; oleic acid, 0.05;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{CaCO}_3$ , 0.05;  $\text{Na}_2\text{CO}_3$ , 0.05; *D*-inositol, 0.04; guanine, 0.05; xanthine, 0.05; uracil,† 0.1; guanidine-HCl, 0.05;  $\text{CuCl}_2$ , 0.0001; 85%  $\text{H}_2\text{MoO}_4$ , 0.00001;  $\text{H}_3\text{BO}_3$ , 0.0005;  $\text{MnSO}_4$ , 0.001;  $\text{ZnCl}_2$ , 0.0005;  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ , 0.0005. pH  $6.5 \pm 0.1$ .

\* Vitamin mixture ( $\mu\text{g}/\text{ml}$ ): nicotinic acid, 7.0; Ca-pantothenate, 2.5; d-riboflavin, 2.5; thiamine-HCl, 2.0; pyridoxine, 1.0; biotin, 0.01.

† Synthesized by T/5 W. L. Mosby.

In order to determine whether *P. oryzae* can be continuously cultivated in simplified media, the fungus was carried for 6 successive subcultures on a chemically defined agar medium and the growth and conidia production compared with 6 corresponding transfers on a 2 per cent rice polish, 2 per cent agar medium. The degree of variation in sporulation on subculture in the two types of media was analyzed statistically. The results are presented in table 3. It is apparent that a good chemically defined medium supports adequate sporulation with less variation on subculture than does the natural medium.

*Nitrogen requirements.* The requirements of *P. oryzae* for amino nitrogen were studied by the omission of each amino acid from a medium containing a mixture of 15 amino acids. The mixture contained glycine, *l*(+)-lysine, *dl*-valine,

*l*(-)-leucine, *dl*-isoleucine, *dl*-threonine, *dl*-phenylalanine, *dl*-methionine, *dl*-glutamic acid, *dl*-aspartic acid, *l*(-)-proline, *l*(-)-hydroxyproline, *l*(+)-arginine, *l*(-)-tryptophane, and *l*(+)-histidine in concentrations equivalent to their pro-

TABLE 2

*Determination of optimal biotin level for growth and conidia production by P. oryzae*

BIOTIN (μg/ML)	SPORE COUNT IN THOUSANDS PER CM <sup>2</sup>	VISUAL ESTIMATION OF GROWTH
0.0	0	none
0.00001	0	very slight
0.0001	4.2	poor
0.001	1,220	good
0.01	1,880	good
0.02	1,610	good

Basal medium (g/L): glucose, 5.0; acid-hydrolyzed "vitamin-free" casein, 1.0; agar, 20; 62% potassium glycerol phosphate, 0.9; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; *i*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine·HCl, 0.0066; choline·Cl, 0.001; thiamine·HCl, 0.002; CuCl<sub>2</sub>, 0.0001; 85% H<sub>2</sub>MoO<sub>4</sub>, 0.0001; H<sub>3</sub>BO<sub>3</sub>, 0.0005; MnSO<sub>4</sub>, 0.001; ZnCl<sub>2</sub>, 0.0005; Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.0005. pH 6.5 ± 0.1.

TABLE 3

*Comparison of variations of conidia production upon subculture of P. oryzae on a rice polish medium and a chemically defined medium*

SUBCULTURE NO.	2% RICE POLISH, 2% AGAR MEDIUM CONIDIA IN THOUSANDS/CM <sup>2</sup>	CHEMICALLY DEFINED MEDIUM* CONIDIA IN THOUSANDS/CM <sup>2</sup>
1	1,700	896
2	771	1,250
3	1,860	615
4	2,720	844
5	760	760
6	292	781
Total.....	8,100	5,150
Average.....	1,350	858
Mean deviation between subcultures.....	66.3%	24.9%

\* Medium (g/L): glucose, 5.0; agar, 20; K<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; CaCl<sub>2</sub>, 0.05; Na<sub>2</sub>CO<sub>3</sub>, 0.05; glycerol, 0.05; thiamine·HCl, 0.002; choline·Cl, 0.001; biotin, 0.00001; *i*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine·HCl, 0.0066; *l*(-)-tryptophane, 0.0193 (0.0001 M); *dl*-glutamic acid, 0.0144 (0.0001 M); *l*(-)-leucine, 0.013 (0.0001 M); *l*(-)-proline, 0.0117 (0.0001 M); *l*(+)-histidine, 0.0154 (0.0001 M); glycine, 0.788 (to raise amino nitrogen to level equivalent to 0.1% casein); CuCl<sub>2</sub>, 0.0001; 85% H<sub>2</sub>MoO<sub>4</sub>, 0.00001; H<sub>3</sub>BO<sub>3</sub>, 0.0005; MnSO<sub>4</sub>, 0.001; ZnCl<sub>2</sub>, 0.0005; Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.0005. pH 6.5 ± 0.1.

portions in 0.1 per cent casein. The single omission of each of the amino acids from the medium made little difference in growth or conidia production. In later work it became apparent that any one of several amino acids could function equally well as a nitrogen source, provided a concentration at least equal to the

amino nitrogen of 0.1 per cent casein was used. The results of this work, including 6 successive subcultures, are presented in table 4. It is evident that *P. oryzae* can be maintained in subculture in media such as those given in table 4.

TABLE 4

*Effects of various amino acids and of  $(\text{NH}_4)_2\text{SO}_4$  as nitrogen sources in the continuous cultivation of *P. oryzae**

NITROGEN SOURCE	c/L	SPORES IN THOUSANDS/CM <sup>2</sup>					
		Subculture no.					
		1	2	3	4	5	6
Casein	1.0	310	247	55.3	101	223	111
$(\text{NH}_4)_2\text{SO}_4^*$	0.75	4.2	7.0	2.1	2.1	3.5	5.6
Glycine†	0.86	213	334	56.0	98.7	89.6	244
<i>dl</i> -Tryptophane	0.204	83.3	134	179	56	104	200
Glycine†	0.7						
<i>dl</i> -Glutamic acid	0.147	101	103	128	125	119	120
Glycine†	0.7						
<i>dl</i> -Leucine	0.131	82.6	94.5	129	62.3	76.3	132
Glycine†	0.7						
<i>dl</i> -Tryptophane	0.204						
<i>dl</i> -Glutamic acid	0.147	43.4	82.6	26.6	22.8	35.0	72.8
<i>dl</i> -Leucine	0.131						
Glycine†	0.42						
<i>l</i> (-)-Tryptophane	0.0204						
<i>dl</i> -Glutamic acid	0.0147						
<i>l</i> (-)-Leucine	0.0131	120	179	302	82.6	229	252
<i>l</i> (-)-Proline	0.0115						
<i>l</i> (+)-Histidine	0.0155						
Glycine†	0.79						

Medium (g/L): glucose, 5.0; agar, 20; 62% potassium glycerol phosphate, 0.9;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001;  $\text{CaCl}_2$ , 0.0005;  $\text{Na}_2\text{CO}_3$ , 0.0005; *D*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine-HCl, 0.0066; choline-Cl, 0.001; thiamine-HCl, 0.002; biotin, 0.00001;  $\text{CuCl}_2$ , 0.0001; 85%  $\text{H}_2\text{MoO}_4$ , 0.00001;  $\text{H}_3\text{BO}_3$ , 0.0005;  $\text{MnSO}_4$ , 0.01;  $\text{ZnCl}_2$ , 0.0005;  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ , 0.0005. pH  $6.5 \pm 0.1$ .

\* Conidia produced in  $(\text{NH}_4)_2\text{SO}_4$  media were morphologically abnormal and failed to germinate. Subcultures in  $(\text{NH}_4)_2\text{SO}_4$  media were made by mycelial transfer.

† Glycine was added in each experiment to raise the concentration of amino nitrogen to that of 0.1% casein.

Experiments were then undertaken to determine the various sources of organic nitrogen, other than amino acids, which are available to this fungus. Two basal media were used, one containing no organic nitrogen (except that in  $0.01 \mu\text{g}$  per ml biotin and  $2.0 \mu\text{g}$  per ml thiamine-HCl) and the other containing  $12.5 \mu\text{g}$  per

ml of total organic nitrogen of which 3.7  $\mu\text{g}$  per ml were amino nitrogen. The compounds to be tested were all added to the basal medium in 0.1 per cent concentration. The results are presented in table 5. Although a large number of widely diverse nitrogenous compounds supported growth and conidia formation,

TABLE 5

*Effects of various nitrogen sources on the growth and sporulation of P. oryzae*

TEST COMPOUND*	"NITROGEN-FREE" BASAL MEDIUM†		"NITROGEN-FREE" BASAL MEDIUM PLUS MIXTURE OF NITROGENOUS NUTRIENTS‡		
	Growth	Conidia in thousands/cm <sup>2</sup>	Growth	Conidia in thousands/cm <sup>2</sup>	
				1	2
Casein.....	+++++	790	++++	235	20.0
Glycine.....	++++	113	+++	409	369
Betaine.....	+	0	+++	83.3	61.6
$\epsilon$ -NH <sub>2</sub> -caproic acid.....	+	0	++	31.5	74.2
Guanidine-HCl.....	+	0	+++	83.3	57.4
Urea.....	++	7.0	+++	110	72.8
Thiourea.....	0	0	0	0	0
Nicotinic acid.....	+	0	++	41.3	46.2
<i>p</i> -NH <sub>2</sub> -benzoic acid.....	+	0	++	0	3.5
Uracil.....	++	7.0	+++	10.5	55.3
Uric acid.....	+++	14.0	+++	67.9	120
Caffeine (citratd).....	+	0	++	78.4	32.2
Triethanolamine.....	+	0	+++	34.3	63.0
Ethanolamine.....	+	0	+++	20.3	35.0
Choline chloride.....	+	0	+++	44.8	36.4
Hydroxylamine.....	0	0	0	0	0
NH <sub>4</sub> Cl.....	0	0	+		9.8
None.....	0	0	+		37.1

\* All compounds except NH<sub>4</sub>Cl were tested at 0.1% concentration. The pH was adjusted to  $6.5 \pm 0.1$  before inoculation of the media. NH<sub>4</sub>Cl was tested at 0.06% concentration, equivalent to 0.1% casein.

† "Nitrogen-free" basal medium (g/L): glucose, 5.0; 62% potassium glycerophosphate, 0.9; agar, 20; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; CaCO<sub>3</sub>, 0.05; Na<sub>2</sub>CO<sub>3</sub>, 0.05; *i*-inositol, 0.02; thiamine-HCl, 0.002; biotin, 0.00001; CuCl<sub>2</sub>, 0.0001; 85% H<sub>2</sub>MoO<sub>4</sub>, 0.00001; H<sub>3</sub>BO<sub>3</sub>, 0.0005; MnSO<sub>4</sub>, 0.001; ZnCl<sub>2</sub>, 0.0005; Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.0005. pH  $6.5 \pm 0.1$ .

‡ Mixtures of nitrogenous nutrients ( $\mu\text{g}/\text{ml}$  in final medium): guanine, 6.6; xanthine, 6.6; uracil, 6.6; guanidine-HCl, 6.6; choline-Cl, 1.0.

$\alpha$ -amino acids were required for full activity. The amino acid requirement was apparently satisfied by glycine alone.

With the medium (table 4) containing the six amino acids (glycine, tryptophane, histidine, leucine, proline, and glutamic acid), choline, inositol, guanine, guanidine, uracil, and xanthine were omitted from the medium singly and as a group to determine whether any of these compounds are essential in an amino acid basal medium. The results (table 6) indicate that none of these compounds are essential for growth or conidia production during a period of 4 subcultures.



Some of the compounds named may be beneficial in stabilizing the growth and conidia production of the organism.

Some of the media were altered for use in submerged cultures by reducing the content of agar to 0.1 per cent. Heavy growth was observed, but conidia were not formed in liquid cultures incubated in aerated bottles. No attempts were made to induce conidia formation in submerged cultures by nutritional alterations. A shaking machine for aeration by agitation was not available during this work; its use might prove valuable in producing submerged sporulated cultures.

TABLE 6

*Effects of certain accessory growth factors on growth and production of conidia by P. oryzae*

COMPOUND OMITTED FROM MEDIUM	SPORES IN THOUSANDS/CM <sup>2</sup>			
	Subculture no.			
	1	2	3	4
None.....	222	1,420	362	998
Choline·Cl.....	78.4	778	1,350	511
Inositol.....	81.2	491	314	330
Guanidine·HCl.....	109	312	167	360
Guanine.....	95.2	225	1,450	750
Xanthine.....	104	557	1,130	213
Uracil.....	137	878	918	692
Choline·Cl	146	347	1,280	943
Inositol				
Guanine				
Xanthine				
Uracil				
Guanidine·HCl				

Medium: Same as given in table 4 including the six amino acids listed together. The compounds named above were used in the concentrations given in table 4.

Conidia of *P. oryzae* produced in chemically defined media have shown 97 to 99 per cent germination<sup>7</sup> and have been found as infective<sup>8</sup> for the rice plant in the greenhouse as conidia produced on natural media.

#### DISCUSSION

On the basis of these studies, the nutritional requirements of *P. oryzae* appear to be relatively nonspecific except with regard to the vitamins, thiamine and biotin being the only ones required. This fungus requires organically combined nitrogen (preferably  $\alpha$ -amino acids), but a large number of compounds in which the nitrogen exists in amino, cyclic, imino, or quaternary combination will support growth and conidia formation. No complete investigations were made of the essentiality of some of the other components of the medium, especially the inorganic ions.

<sup>7</sup> We wish to thank Capt. J. W. Marek, AUS, for performing the germination experiments.

<sup>8</sup> We wish to thank S/Sgt. T. L. Morgan, AUS, for performing the infectivity experiments.

Although the fungus grew more uniformly on the chemically defined media than on natural media, such as rice polish agar, the degree of variation was considerable with all media. The variability of the quantity of growth in replicates prevents accurate evaluation of nutrients the effects of which are quantitatively of a low order. Perhaps further nutritional investigations would lead to greater uniformity, especially if submerged dispersed growth in liquid media could be used in place of surface growth. Although the production of conidia was less variable on an adequate chemically defined medium than on a natural medium (table 3), when certain of the pure nutrient compounds were omitted from chemically defined media to determine their essentiality (tables 4 and 6) the resulting cultures were sometimes as variable as those on rice polish agar.

#### SUMMARY

Thiamine (2  $\mu\text{g}$  per ml or less) and biotin (0.01  $\mu\text{g}$  per ml) are required for growth and conidia formation by *Piricularia oryzae*. Other B complex vitamins are not required.

*P. oryzae* requires organically combined nitrogen, preferably  $\alpha$ -amino acids, but can use many types of organic compounds in which the nitrogen exists in amino, imino, cyclic, or quaternary combination.

*P. oryzae* can be maintained successfully in subculture on chemically defined media, the degree of variation and the yields, viability, and the degree of germination and infectivity of conidia comparing favorably with cultures grown on natural media.

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# THE TUBERCULOSTATIC ACTION OF PARA-AMINOSALICYLIC ACID<sup>1</sup>

GUY P. YOUMANS, GORDON W. RALEIGH, AND ANNE S. YOUMANS

*Department of Bacteriology and Department of Pathology, Northwestern University Medical School, Chicago 11, Illinois, and the Evanston Hospital, Evanston, Illinois*

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Bernheim (1941, 1942) reported that sodium benzoate and sodium salicylate increased the oxygen uptake of tubercle bacilli. Lehman (1946a) reported that the respiration of virulent tubercle bacilli was stimulated by sodium salicylate but that the respiration of avirulent tubercle bacilli was not. On the basis of this work Lehman (1946b), attempting to find a substance which would be bacteriostatic to tubercle bacilli, tested more than 50 derivatives of benzoic acid for their bacteriostatic activity. The most active substance found was *para*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid—PAS). This substance was bacteriostatic in a concentration of 0.15 mg per 100 ml for the BCG strain of the bovine type of *Mycobacterium tuberculosis* and, according to Lehman, exerted a favorable effect on clinical tuberculosis. In a more recent communication, Lehman (1946c) amplified his earlier work and apparently observed some retardation of experimental tuberculosis of guinea pigs after 7 days' treatment with PAS.

Youmans (1946), in a preliminary communication, reported that PAS was highly bacteriostatic for 12 virulent strains of human type tubercle bacilli and exerted a suppressive action on experimental tuberculosis of mice.

The present paper details further work on the tuberculostatic activity of *p*-aminosalicylic acid both *in vitro* and *in vivo*.

## METHODS

The tuberculostatic activity of PAS<sup>2</sup> was tested *in vitro* by the method described previously (Youmans, 1944; Youmans and Doub, 1946) by determining the least amount which would completely inhibit the subsurface growth of 0.01 mg of tubercle bacilli per ml of synthetic medium. These tuberculostatic tests were also done in synthetic medium to which was added enough bovine serum to make a final concentration of 10 per cent. The effect of sodium salicylate and *para*-aminobenzoic acid (PABA) on the bacteriostatic power of PAS was tested by incorporating the former substances in media in which dilutions of PAS were prepared. The test materials were incubated at 37 C, and the results were read at the end of 14 days. The human type strains of tubercle bacilli tested had been isolated from patients with tuberculosis within the preceding

<sup>1</sup> This work was aided by a Research Grant from Parke, Davis & Company, Detroit, Michigan.

<sup>2</sup> Prepared for this purpose by Leonard Doub and Martin L. Black of the Research Laboratories, Parke, Davis & Company, Detroit.

year and a half, with the exception of the standard H37Rv strain. In addition, one stock bovine type strain and one avian type, as well as the avirulent rapidly growing strain no. 607, were used. Six of the human strains were streptomycin-resistant (Williston and Youmans, 1947).

The effect of PAS *in vivo* was determined by infecting mice<sup>3</sup> intravenously with 0.1 mg of the H37Rv strain as previously described (Youmans and McCarter, 1946; Youmans and Williston, 1946). PAS was incorporated in the desired concentration in the mouse diet which consisted of a powdered food obtained by grinding Rockland Farms complete mouse ration pellets and passing them through a sieve in order to eliminate coarse particles. PAS was used in these mouse experiments in the form of both the hydrochloride and the free base; with the latter compound one mole of sodium bicarbonate was added for each mole of PAS. The mice were fed the diets containing PAS for 28 days, starting the day before they were infected with tubercle bacilli.

In untreated control and corresponding treated series, animals remaining alive at the end of 28 days were sacrificed by exposure to ether vapor, weighed, and eviscerated. At that time, random tissue specimens were removed and cultured on Herrold's glycerol egg yolk agar and a variety of other media designed to grow most types of organisms. The viscera were then fixed *in toto* in 3.7 per cent formaldehyde solution. Subsequently they were dissected and only the larger parenchymatous organs, i.e., lungs, livers, kidneys, and spleens, were retained. These were re-examined after fixation, and an evaluation of the extent of the lesions was made on the following basis: zero (0), apparently normal; plus or minus ( $\pm$ ), no definite lesions but a questionable deviation from normal; one plus sign (+), less than 10 per cent involvement; two plus signs (++), from 10 to 25 per cent involvement; three plus signs (+++), 25 to 50 per cent involvement; and four plus signs (++++), 50 to 75 per cent, or possibly more, of the organ composed of grossly pathologic tissue. Notation was also made of the predominant type of lesion, tuberculoid or patchy, present in the lungs. Because previous experience in this laboratory had indicated a high degree of constancy in the lesions produced by this method, only sample tissues were selected for sectioning from animals in which the greatest or least pathologic change seemed probable. These tissues were dehydrated and embedded in paraffin according to the usual technique, and sections were cut at five microns. Each was stained with hemotoxylin and eosin and by the Ziehl-Neelsen carbol fuchsin technique for acid-fast organisms. The extent of microscopic involvement was tabulated on the following basis: zero (0), apparently normal; one plus sign (+), less than 10 per cent of the organ involved; two plus signs (++), 10 to 25 per cent replaced; three plus signs (+++), 25 to 50 per cent involved; and four plus signs (++++), 50 to 75 per cent, or possibly more, of the organ replaced by pathologic tissue. Notation was made of the predominant type of lesion present, necrotic-exudative or proliferative. Sections stained for acid-fast bacilli and studied by oil immersion were graded on the following basis: zero (0), no acid-fast bacilli seen; one plus sign (+), a few single, or clumps of 4 or 5,

<sup>3</sup> Strong A strain.

intra- or extra-cellular organisms observed in occasional fields; two plus signs (++) , moderate number of single organisms or groups of 4 or 5, observed intra- or extra-cellularly in about half of the fields; three plus signs (+++) , solitary bacilli or clumps of intra- or extra-cellular organisms seen in more than half of the fields; and four plus signs (++++), single organisms, clumps, and large masses of bacilli found within and outside cells in more than half the fields.

Experiments were also conducted in which both streptomycin and PAS were administered to mice infected with tubercle bacilli. In these experiments the

TABLE 1

*Bacteriostatic effect of p-amino salicylic acid on streptomycin-sensitive and streptomycin-resistant strains of tubercle bacilli*

STRAIN NO.	TYPE	CONCENTRATION IN MG PER 100 ML WHICH COMPLETELY INHIBITED GROWTH	
		Without plasma	With plasma
H37Rv	Human	0.078	0.156
H37RvR*	Human	0.039	0.039
100	Human	0.078	
100R	Human	0.019	
23	Human	0.039	0.156
23R	Human	0.039	0.078
24	Human	0.019	0.039
24R	Human	0.019	0.019
69	Human	0.019	0.039
69R	Human	0.039	0.078
97	Human	0.0095	0.019
97R	Human	0.019	0.019
111	Human	0.156	
1	Human	0.078	0.156
11	Human	0.039	0.039
12	Human	0.078	0.078
18	Human	0.019	0.019
48	Bovine	No growth	0.039
37	Avian	0.625	0.625
607	?	>100.0	

\* R indicates a streptomycin-resistant strain.

PAS was administered in the diet as before, whereas streptomycin in distilled water was given in 2 daily subcutaneous injections of 0.2 ml each, 8 hours apart.

#### RESULTS

Table 1 shows the *in vitro* bacteriostatic effect of PAS on the strains of tubercle bacilli employed. All of the strains except one were inhibited by very low concentrations of PAS. The human strains appeared to be approximately equally sensitive to the bacteriostatic activity of PAS, and furthermore this activity was not markedly affected by the presence of 10 per cent bovine plasma. There was no significant difference between the results obtained with the streptomycin-sensitive and resistant strains. The one bovine strain appeared to be as sus-

ceptible as the human strains, whereas the one avian strain seemed to be slightly more resistant to the bacteriostatic activity of PAS. The rapidly growing avirulent strain no. 607, however, was highly resistant to the bacteriostatic activity of PAS, growth occurring even in a concentration of 100 mg per cent.

It should also be noted that the bacteriostatic activity of PAS for human type tubercle bacilli was of approximately the same order as that of streptomycin, since in most cases less than 1 microgram per ml of medium completely inhibited growth.

Table 2 shows the effect of the number of organisms in the inoculum on the *in vitro* bacteriostatic activity of PAS. The bacteriostatic activity of this compound, as has been observed with the sulfonamides and the sulfones, is markedly affected by the number of organisms present. However, even with inocula which in this experiment gave growth in a concentration of 10 mg per cent there was still partial bacteriostatic activity in a concentration of 0.156 mg per cent, as determined by comparing the growth in these tubes with the control.

TABLE 2

*The effect of the number of tubercle bacilli (H37Rv) upon the bacteriostatic activity of PAS*

NO. OF TUBERCLE BACILLI IN MG PER ML OF MEDIUM	CONCENTRATION OF PAS										Controls
	10.0	5.0	2.5	1.25	0.625	0.312	0.156	0.078	0.039	0.019	
0.01	—	—	—	—	—	—	—	—	—	S	G
0.02	—	—	—	—	—	—	—	—	S	S	G
0.03	—	—	—	—	—	—	S	S	M	G	G
0.04	—	—	—	S	S	S	M	M	G	G	G
0.05	S	S	S	S	S	M	M	G	G	G	G

— = no growth; S = slight growth; M = moderate growth; G = good growth.

When PAS was tested in a medium containing 0.1 mg PABA per 100 ml, the bacteriostatic activity was reduced to one-sixteenth of its former value. Although this reduction of bacteriostatic activity is not great, it possibly indicates that the bacteriostatic action is, at least in part, due to anti-PABA action.

If the activity of PAS is due to its antisalicylate effect, as implied by Lehman (1946b), one would expect sodium salicylate to have some anti-PAS action. However, the bacteriostatic activity of PAS was not influenced by the presence of sodium salicylate in the medium in concentrations of 0.1 and 1.0 mg per 100 ml. Lehman (1947) also failed to observe any interference by PAS with the stimulation of respiration by salicylates.

The combined bacteriostatic activity of PAS and streptomycin *in vitro* was also tested. In all cases, however, the degree of bacteriostasis was no greater than the sum of the individual activities of the two substances.

Table 3 shows the *in vitro* bacteriostatic activity of 13 derivatives of PAS and salicylic acid.<sup>4</sup> In all cases the bacteriostatic activity is markedly less than that of PAS.

<sup>4</sup> These compounds prepared by Leonard Doub and Dr. L. L. Bambas, Research Laboratories, Parke, Davis & Company, Detroit, Michigan.

Referring to table 4, it will be noted that inclusion of a 2 per cent concentration of *para*-aminosalicylic acid in the diet prolonged the average survival time of mice infected with 0.1 mg of H37Rv to 27.9 days as compared with the average survival time of 20.2 days for the control series. Inasmuch as all the treated animals but two survived the time limit of the experiment, and one of these was accidentally killed, it is probable that the actual differential is greater than that apparent. Although the treated animals did not experience the expected weight gain for their age (about 5 to 10 grams in 4 weeks), the average final weight was about equal to that recorded initially. This compares favorably with the average weight loss of 5.8 grams for the control animals. Again it will be noted that the treated animals averaged 1.7+ gross pulmonary involvement

TABLE 3

*Bacteriostatic activity of derivatives of p-aminosalicylic acid and derivatives of salicylic acid on M. tuberculosis (H37Rv)*

COMPOUND	LEAST AMOUNT IN MG/100 ML WHICH COMPLETELY INHIBITS GROWTH OF M. H37Rv
<i>p</i> -Aminosalicylic acid .....	0.039-0.078
5-Amino-2-hydroxybenzoic acid.....	>10.0
4-Amino-2-hydroxybenzamide.....	10.0
Ethyl-4-amino-2-hydroxy benzoate.....	2.5
4-Amino-2-methoxybenzoic acid .....	>10.0
4-Acetyl-amino-2-hydroxybenzoic acid.....	10.0
<i>m</i> -Aminophenol.....	>10.0
Salicylic acid .....	10.0
2-Methoxybenzoic acid.....	>10.0
2-Hydroxybenzyl alcohol.....	10.0
3-Hydroxybenzoic acid.....	>10.0
4-Amino-2-hydroxybenzene sulfonic acid.....	>10.0
Thiosalicylic acid .....	5.0
4-Amino-2-hydroxybenzene arsonic acid.....	5.0

as compared with the average of 3.8+ in the control series. Qualitative histopathologic changes in the treated and untreated groups were related in reciprocal manner. Thus the majority of the control animals exhibited greatly enlarged, tubercle-bearing lungs, the unit lesion of which was of necrotic-exudative character with large numbers of bacilli. Conversely, the majority of the treated animals' lungs were smaller and were the site of proliferative changes with fewer mycobacteria present. These findings suggest a lack of general tissue toxicity or depression of the defensive mechanism. The predominance of the proliferative lesions at prolonged survival times, hence longer evolution times for the unit lesions, implies a depressive influence exerted on the bacteria or a stimulating influence on resistance. In view of the *in vitro* effectiveness of this compound it seems logical to assume that at least the greater portion of this effect was exerted upon the bacteria directly.

Increasing the concentration of the drug to 4 per cent in the diet resulted in a

reduction of average survival time to 14.4 days and the disproportionately great average weight loss of 6.3 grams for this short survival period. The histopathology of the unit lesions was in keeping with the short survival time. These data indicate a toxic effect exerted by the drug on the body tissues in general.

When PAS was employed in a 1 per cent concentration in the diet, survival time was prolonged to the limits of the experiment, whereas the average control animal survived 20.8 days. Here again, the actual differential is probably greater than that apparent. The treated animals experienced an average weight gain of 1.2 grams as compared with the average loss of 5.1 grams for the control series. Whereas the extent of gross pulmonary change in the treated animals averaged 2.9+ as compared with the average of 3.8+ for the control series, the

TABLE 4  
*Effect of p-aminosalicylic acid on experimental tuberculosis of white mice*

NUMBER OF MICE	PER CENT PAS IN DIET	NUMBER DEAD	PER CENT MORTALITY	AVERAGE SURVIVAL TIME	AVERAGE WEIGHT LOSS OR GAIN	AVERAGE AMOUNT GROSS PULMONARY TUBERCULOSIS
<i>p</i> -Aminosalicylic acid hydrochloride						
20	0.0	17	85.0	20.2	-5.8	3.8+
19*	2.0	1	5.26	27.9	+0.4	1.7+
20	4.0	13	65.0	14.4	-6.3	1.2+
20	0.0	15	75.0	20.8	-5.1	3.9+
20	1.0	0	0.0	28.0	+1.2	2.9+
<i>p</i> -Aminosalicylic acid (free base)						
15	0.0	12	80.0	23.6	-5.2	3.8+
15	1.0	0	0.0	28.0	+1.0	2.4+
15	2.0	0	0.0	28.0	-1.2	1.6+
15	4.0	12	80.0	13.2		2.4+

\* One mouse killed accidentally and not included.

proliferative histopathologic pattern, with low bacterial concentrations, predominated in the former group. These data follow the same pattern as those determined with the 2 per cent concentration. Since the control data for both of these series are closely parallel, comparison of the two treated groups seems valid. It is apparent that the 1 per cent concentration permitted a somewhat more extensive pulmonary change, still of chiefly proliferative pattern, and a greater weight gain. This seeming paradox might be construed as evidence that the 2 per cent concentration was slightly toxic, even though evidence of its toxicity was not previously adduced, and that the 1 per cent concentration exerted somewhat less depressive action on the bacteria.

Since it was felt that the toxicity observed in these experiments might be due to the acidity of the compound when used in the form of the hydrochloride, the next experiment was done employing PAS in the form of the free base, to which



was added sodium bicarbonate. The results, however, were in every respect similar to the first experiment both as to survival time and the extent of the gross and microscopic lesions in the lungs (table 4). Tubercle bacilli were recovered by culture from both control and PAS-treated animals, but in fewer numbers from the latter animals. No other types of bacteria were isolated.

From these results it is apparent that PAS is moderately effective in suppressing experimental pulmonary tuberculosis of mice but is toxic in these animals in concentrations of over 1 per cent. Lehman (1947) states that mice tolerate well concentrations of this drug up to 5 per cent. The reason for this discrepancy in our results is not apparent unless the diets which were different in Lehman's experiments than in ours might have influenced the toxic reactions obtained.

Previous experience in this laboratory with similarly conducted experiments (Youmans and McCarter, 1946) has demonstrated the effectiveness of streptomycin therapy in prolonging the survival time and reducing the weight loss of the experimental animals, as well as in the reduction of the extent of the pathologic change in the organs and the concentration of bacteria in the unit lesions. Further experience with that antibiotic agent has demonstrated a correlated qualitative change in that the pulmonary lesions of the treated animals were predominantly of the proliferative type, whereas those of the control groups are principally of the necrotic-exudative type. In the experience of the present writers, these favorable patterns are somewhat more impressive when streptomycin is employed in optimum subcutaneous dosage than when *para*-aminosalicylic acid is added to the diet in either 1 or 2 per cent concentration.

Preliminary work, however, indicates that tuberculous mice treated with both streptomycin and PAS show a therapeutic response greater than that observed with either substance alone. As the effect appears to be no more than additive, the implications in the treatment of clinical tuberculosis are obvious. These results will be reported in detail in a subsequent communication.

#### SUMMARY

*Para*-aminosalicylic acid was found to be highly bacteriostatic *in vitro* for virulent human type tubercle bacilli, and this activity was not appreciably affected by the presence of serum in the medium. The bacteriostatic activity was partially reversed by *para*-aminobenzoic acid but was not reversed by sodium salicylate. Furthermore, the bacteriostatic activity of this compound (PAS) was inversely proportional to the number of organisms present in the medium. One avirulent, rapidly growing, acid-fast organism, no. 607, was not inhibited by 100 mg per cent *para*-aminosalicylic acid.

Thirteen derivatives of PAS and salicylic acid were found to be much less tuberculostatic than PAS.

Experimental tuberculosis of mice was found to be suppressed by *para*-aminosalicylic acid when it was administered in the diet in 1 and 2 per cent concentrations, both when the drug was given in the form of the hydrochloride and in the form of free base. Under the conditions of the experiment, 4 per cent *para*-

aminosalicylic acid when administered to mice was highly toxic, whereas 2 per cent was slightly toxic.

PAS and streptomycin when administered to mice simultaneously appeared to exert a suppressive effect on the tuberculous process greater than that of either substance alone.

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# THE BACTERIOSTATIC ACTIVITY OF CERIUM, LANTHANUM, AND THALLIUM

SUDIE BURKES AND C. S. McCLESKEY

*Louisiana State University, Baton Rouge, Louisiana*

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The salts of cerium, lanthanum, and thallium have long been known to possess bacteriostatic properties in low concentrations, but there is little information in the literature concerning the variability of their toxicity toward different species of microorganisms. A report of the concentrations of the salts limiting development of various types of bacteria may have importance in relation to their possible use as bacteriostatic or bactericidal agents.

Bokorny (1894) found cerium compounds relatively much more toxic for bacteria than for algae. Hebert (1907) reported that cerium and lanthanum sulfates in concentrations of 5 to 10 grams per liter showed little toxicity toward *Aspergillus niger* and yeast. Sartory and Bailly (1922) reported that 0.2 per cent lanthanum sulfate depressed the growth of *Aspergillus fumigatus* in Raulin's solution and practically inhibited spore formation. Frouin (1912) observed that 0.005 grams of lanthanum sulfate per 100 ml of medium stimulated the growth of the tubercle bacillus but that higher concentrations were toxic. Frouin and Roudski (1914) studied the toxicity of lanthanum and thorium for the cholera and dysentery organisms.

Other investigators who reported bacteriostatic or lethal effects of salts of the rare earth group include Drossback (1897), Brooks (1921), Grenet and Drouin (1927), Zirpolo (1924), Frouin (1920), Simonini (1914), Doerr (1920), Eisenberg (1918), and Hotchkiss (1923). A general review of the earlier literature on this subject is found in Buchanan and Fulmer (1930). McKenzie (1941) employed thallium acetate in a medium recommended for the enrichment of the streptococci causing mastitis. The effect of cerium on enzyme activity was reported by Gould (1936). Olszewski (1932) observed no significant reduction in the bacteria of river water when 1 ppm of cerous or ceric chloride or ceric sulfate was employed. Richards (1932) reported thallium to be a growth stimulant for yeast.

The present paper reports a further investigation of the bacteriostatic activity of the salts of cerium, lanthanum, and thallium.

## METHODS

Thirty-nine species of bacteria, representing 16 different genera, were employed in this study. Also, 35 species of fungi, comprising 18 genera, were used in a limited comparison of the mycostatic and bacteriostatic effects of the compounds. The salts used were cerium chloride ( $\text{CeCl}_3$ , cp, E. H. Sargent); cerium nitrate ( $\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ , cp, General Chemical Co.); anhydrous ceric sulfate ( $\text{Ce}(\text{SO}_4)_2$ , G. F. Smith Chemical Co.); lanthanum chloride (cp, E. H. Sargent), and thallium nitrate (E. H. Sargent).

Other chemicals were used in some of the tests in order to determine their effect on the toxicity of the test substances. These chemicals included the sulfates and chlorides of sodium, magnesium, barium, and ammonium, and the chlorides of calcium and lithium. Stock solutions of the salts were made and the amount required for each test medium was removed by pipette. The tests were made on petri plates of solidified agar containing the specified amounts of the salts. The basal medium consisted of 1 per cent Difco peptone and 1.5 per cent Difco agar in distilled water. Inoculations of the agar plates were made by means of a 1½-mm nichrome wire loop using a 24-hour broth culture. Radial streak inoculations were made, using 8 cultures to each plate. Incubation was at 37 C except for those soil and water forms which grow better at a lower temperature and were incubated at room temperature (22 to 27 C). Observations and records were made after incubation for 1, 2, 3, and 5 days.

#### RESULTS

*The toxicity of cerium salts for bacteria.* The bacteriostatic action of three cerium salts, the trivalent cerium chloride and cerium nitrate and the tetravalent ceric sulfate, was determined against 40 different species. The results are presented in table 1. The chloride was found to be definitely less toxic than either the sulfate or nitrate of cerium. No significant difference in toxicity between the sulfate and nitrate of cerium was noted.

The reaction of the media was not adjusted after the addition of the cerium salts, and the pH values were found to vary as follows: for cerium chloride agar, 6.3 at 0.0002 M concentration to 5.95 at 0.0014 M concentration; for cerium nitrate, pH 5.8 at 0.0001 M to 5.65 at 0.0009 M; and for cerium sulfate, pH 6.7 at 0.0002 M to 5.4 at 0.0008 M.

The toxicity of cerium nitrate and cerium chloride varied relatively little among the different species of bacteria tested, and even fewer variations were observed with cerium sulfate. Of the bacterial species tried, *Aerobacter aerogenes*, *Aerobacter cloacae*, *Salmonella aertrycke*, and *Achromobacter lipolyticum* were most tolerant of cerium. The *Torula rosea* culture proved to be far more resistant to the cerium compounds than the most resistant bacteria.

*The effect of pH and the presence of other salts on the toxicity of cerium compounds.* In order to determine the effect of pH on cerium toxicity, the media were prepared using cerium nitrate in concentrations varying from 0.0003 M to 0.0007 M, then adjusted to pH 6.0 and 8.0. The results obtained after 2 days' incubation are presented in table 2. It was observed that at pH 8.0 all cultures except *Staphylococcus albus* developed without hindrance in 0.0007 M cerium nitrate, whereas at pH 6.0 a considerable number of cultures failed to grow in the 0.0003 M concentration of cerium.

*The effect of other salts on the bacteriostatic activity of cerium compounds.* The effect of various salts which are sometimes used in culture media on the bacteriostatic activity of cerium compounds was determined by adding the salts separately to the cerium-containing media and observing for bacterial growth after inoculations. It was found that sodium chloride in concentrations up to

TABLE 1  
The toxicity of various cerium compounds for certain bacteria  
Incubation for 2 days

CULTURE	MOLECULAR CONCENTRATION					
	CeCl <sub>3</sub>		Ce(NO <sub>3</sub> ) <sub>3</sub>		Ce(SO <sub>4</sub> ) <sub>2</sub>	
	A	B	A	B	A	B
<i>Salmonella paratyphi</i> .....	0.0006	0.0008	0.0004	0.0005	0.0004	0.0006
<i>Salmonella pullorum</i> .....	0.0006	0.0008	0.0004	0.0005	0.0004	0.0006
<i>Salmonella schottmuelleri</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Salmonella enteritidis</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Salmonella aertrycke</i> .....	0.0008	0.0010	0.0004	0.0005	0.0006	0.0008
<i>Salmonella gallinarum</i> .....	0.0006	0.0008	0.0004	0.0005	0.0004	0.0006
<i>Salmonella suispestifer</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Eberthella typhosa</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Shigella sonnei</i> .....	0.0008	0.0010	0.0005	0.0006	0.0004	0.0006
<i>Shigella dysenteriae</i> .....	0.0008	0.0010	0.0003	0.0004	0.0004	0.0006
<i>Aerobacter acrogenes</i> .....	0.0008	0.0010	0.0005	0.0006	0.0006	0.0008
<i>Aerobacter cloacae</i> .....	†	†	0.0005	0.0006	0.0006	0.0008
<i>Escherichia coli</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Escherichia communior</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Escherichia acidilactici</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Citrobacter intermedium</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Alcaligenes faecalis</i> .....	0.0010	0.0012	0.0004	0.0005	0.0004	0.0006
<i>Proteus vulgaris</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Pseudomonas aeruginosa</i> .....	0.0004	0.0006	0.0003	0.0004	0.0004	0.0006
<i>Pseudomonas ovalis</i> .....	0.0006	0.0008	0.0003	0.0004	0.0004	0.0006
<i>Pseudomonas graveolens</i> .....	0.0004	0.0006	0.0001	0.0002	0.0004	0.0006
<i>Pseudomonas syncyanea</i> .....	0.0006	0.0008	0.0003	0.0004	0.0004	0.0006
<i>Pseudomonas mucedolens</i> .....	0.0004	0.0006	0.0001	0.0002	0.0004	0.0006
<i>Flavobacterium saueolens</i> .....	0.0010	0.0012	0.0005	0.0006	0.0004	0.0006
<i>Achromobacter lipolyticum</i> .....	0.0010	0.0012	0.0006	0.0007	0.0006	0.0008
<i>Serratia marcescens</i> .....	0.0010	0.0012	0.0006	0.0007	0.0004	0.0006
<i>Bacillus subtilis</i> .....	0.0010	0.0012	0.0006	0.0007	0.0004	0.0006
<i>Bacillus mesentericus</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Bacillus mycoides</i> .....	0.0008	0.0010	0.0005	0.0006	0.0004	0.0006
<i>Bacillus fusiformis</i> .....	0.0008	0.0010	0.0004	0.0005	0.0004	0.0006
<i>Bacillus metiens</i> .....	0.0008	0.0010	0.0005	0.0006	0.0004	0.0006
<i>Staphylococcus candidus</i> .....	0.0008	0.0010	0.0005	0.0006	0.0004	0.0006
<i>Staphylococcus flavus</i> .....	0.0008	0.0010	0.0003	0.0004	0.0004	0.0006
<i>Staphylococcus aureus</i> .....	0.0008	0.0010	0.0007	0.0008	0.0004	0.0006
<i>Staphylococcus albus</i> .....	0.0010	0.0012	0.0004	0.0005	0.0004	0.0006
<i>Sarcina lutea</i> .....	0.0006	0.0008	0.0004	0.0005	0.0004	0.0006
<i>Sarcina conjunctivae</i> .....	0.0006	0.0008	0.0007	0.0008	0.0004	0.0006
<i>Rhodococcus agilis</i> .....	0.0006	0.0008	0.0003	0.0004	0.0004	0.0006
<i>Rhodococcus rosaceus</i> .....	0.0006	0.0008	0.0003	0.0004	0.0004	0.0006
<i>Torula rosea</i> .....	0.0012	*	0.0009	*	0.0008	*

A = concentration permitting growth in 2 days.

B = concentration inhibiting growth in 2 days.

\* Inhibiting concentration was not determined.

† Concentration less than .0003 M was not employed.

TABLE 2  
The effect of pH on the toxicity of  $Ce(NO_3)_3$  for certain bacteria

CULTURE	MOLECULAR CONCENTRATION			
	pH 6		pH 8	
	A	B	A	B
<i>S. paratyphi</i> .....	0.0003	0.0004	0.0007	*
<i>S. pullorum</i> .....	0.0003	0.0004	0.0007	*
<i>S. schottmuelleri</i> .....	0.0005	0.0007	0.0007	*
<i>S. enteritidis</i> .....	0.0004	0.0005	0.0007	*
<i>S. aertrycke</i> .....	0.0004	0.0005	0.0007	*
<i>S. gallinarum</i> .....	0.0004	0.0005	0.0007	*
<i>S. suispestifer</i> .....	0.0005	0.0006	0.0007	*
<i>E. typhosa</i> .....	0.0004	0.0005	0.0007	*
<i>S. sonnei</i> .....	†	0.0003	0.0007	*
<i>S. dysenteriae</i> .....	0.0003	0.0004	0.0007	*
<i>A. aerogenes</i> .....	0.0007	*	0.0007	*
<i>A. cloacae</i> .....	0.0006	0.0007	0.0007	*
<i>E. coli</i> .....	0.0004	0.0005	0.0007	*
<i>E. communior</i> .....	0.0004	0.0005	0.0007	*
<i>E. acidilactici</i> .....	0.0003	0.0004	0.0007	*
<i>C. intermedium</i> .....	0.0006	0.0007	0.0007	*
<i>A. faecalis</i> .....	0.0003	0.0004	0.0007	*
<i>P. vulgaris</i> .....	0.0003	0.0004	0.0007	*
<i>P. aeruginosa</i> .....	†	0.0003	0.0007	*
<i>P. ovalis</i> .....	†	0.0003	0.0007	*
<i>P. graveolens</i> .....	†	0.0003	0.0007	*
<i>P. syncyanea</i> .....	†	0.0003	0.0007	*
<i>P. mucedolens</i> .....	†	0.0003	0.0007	*
<i>F. suaveolens</i> .....	0.0007	*	0.0007	*
<i>A. lipolyticum</i> .....	0.0007	*	0.0007	*
<i>S. marcescens</i> .....	0.0007	*	0.0007	*
<i>B. subtilis</i> .....	†	0.0003	0.0007	*
<i>B. mesentericus</i> .....	0.0003	0.0004	0.0007	*
<i>B. mycoides</i> .....	†	0.0003	0.0007	*
<i>B. fusiformis</i> .....	†	0.0003	0.0007	*
<i>B. meliens</i> .....	†	0.0003	0.0007	*
<i>S. aureus</i> .....	0.0006	0.0007	0.0007	*
<i>S. candidus</i> .....	0.0004	0.0005	0.0007	*
<i>S. albus</i> .....	0.0003	0.0004	0.0004	0.0005
<i>S. flava</i> .....	0.0003	0.0004	0.0007	*
<i>S. lutea</i> .....	†	0.0003	0.0007	*
<i>S. conjunctivae</i> .....	†	0.0003	0.0007	*
<i>R. agilis</i> .....	0.0003	0.0004	0.0007	*
<i>R. rosaceous</i> .....	†	0.0003	0.0007	*
<i>Torula rosea</i> .....	0.0007	*	0.0007	*

A = concentration permitting growth in 2 days.

B = concentration inhibiting growth in 2 days.

\* Concentration greater than 0.0007 M was not employed.

† Concentration less than 0.0003 M was not employed.

TABLE 3

*The effect of 0.4 M of NaCl and 0.1 M Na<sub>2</sub>SO<sub>4</sub> on toxicity of Ce(SO<sub>4</sub>)<sub>2</sub>*

CULTURE	Ce(SO <sub>4</sub> ) <sub>2</sub> CONC. (M)	CONTROLS FOR			Ce(SO <sub>4</sub> ) <sub>2</sub> PLUS	
		Ce(SO <sub>4</sub> ) <sub>2</sub>	NaCl	Na <sub>2</sub> SO <sub>4</sub>	NaCl	Na <sub>2</sub> SO <sub>4</sub>
		Vigor of growth: incubation for 2 days				
<i>S. paratyphi</i> .....	0.0006	—	++	++	—	+
<i>S. pullorum</i> .....	0.0006	—	++	++	—	++
<i>S. schottmuelleri</i> .....	0.0008	—	+++	+++	—	++
<i>S. enteritidis</i> .....	0.0008	—	++	++	—	+
<i>S. aertrycke</i> .....	0.0008	+	++	++	—	+
<i>S. gallinarum</i> .....	0.0004	+	++	++	++	+
<i>S. suispestifer</i> .....	0.0008	—	++	++	—	+
<i>E. typhosa</i> .....	0.0006	—	+++	+++	—	++
<i>S. sonnei</i> .....	0.0006	—	++	++	—	+
<i>S. dysenteriae</i> .....	0.0004	++	++	++	+	+
<i>A. aerogenes</i> .....	0.0008	+	++	++	—	++
<i>A. cloacae</i> .....	0.0008	++	+++	++	—	++
<i>E. coli</i> .....	0.0006	—	+++	++	—	+
<i>E. communior</i> .....	0.0008	—	+++	+++	—	+
<i>C. intermedium</i> .....	0.0006	—	++	++	—	+
<i>A. faecalis</i> .....	0.0006	—	+++	++	—	+
<i>P. vulgaris</i> .....	0.0006	—	++	++	—	+
<i>P. aeruginosa</i> .....	0.0004	++	++	+++	—	++
<i>P. ovalis</i> .....	0.0004	++	++	++	+	++
<i>P. graveolens</i> .....	0.0004	++	++	++	—	+
<i>P. syncyanea</i> .....	0.0004	+	+++	+++	—	++
<i>P. mucedolens</i> .....	0.0004	+	++	++	—	+
<i>F. suaveolens</i> .....	0.0006	—	++	++	—	++
<i>A. lipolyticum</i> .....	0.0008	+	++	++	+	+
<i>S. marcescens</i> .....	0.0006	+	++	++	+	++
<i>B. subtilis</i> .....	0.0006	+	++	+++	—	++
<i>B. mesentericus</i> .....	0.0008	—	++	+++	—	+
<i>B. mycoides</i> .....	0.0004	++	++	+++	+	++
<i>B. fusiformis</i> .....	0.0004	++	++	+++	++	++
<i>B. metiens</i> .....	0.0004	++	++	++	++	++
<i>S. aureus</i> .....	0.0004	++	++	++	++	++
<i>S. candidus</i> .....	0.0006	—	++	++	—	+
<i>S. albus</i> .....	0.0004	++	++	++	+	++
<i>S. flavus</i> .....	0.0004	++	++	++	+	+
<i>S. lutea</i> .....	0.0004	+	++	++	—	+
<i>S. conjunctivae</i> .....	0.0004	++	++	++	+	++
<i>R. agilis</i> .....	0.0004	+	++	++	+	+
<i>R. rosaceus</i> .....	0.0004	+	++	++	+	+
<i>Torula rosea</i> .....	0.0004	++	++	++	++	++

(-) = complete inhibition of growth.

(+) = moderate growth.

(++) = good growth.

(+++)= growth better than on nutrient agar control.

TABLE 4

*The bacteriostatic activity of lanthanum chloride and thallium nitrate*

ORGANISM	MOLECULAR CONCENTRATION			
	Permitting growth		Preventing growth	
	LaCl <sub>3</sub>	TlNO <sub>3</sub>	LaCl <sub>3</sub>	TlNO <sub>3</sub>
<i>S. paratyphi</i> .....	0.0002	0.0006	0.0004	0.0007
<i>S. pullorum</i> .....	0.0002	0.0006	0.0004	0.0007
<i>S. schottmuelleri</i> .....	0.0004	0.0007	0.0006	0.0008
<i>S. enteritidis</i> .....	0.0004	0.0007	0.0006	0.0008
<i>S. aertrycke</i> .....	0.0004	0.0007	0.0006	0.0008
<i>S. gallinarum</i> .....	0.0004	0.0006	0.0006	0.0007
<i>S. suispestifer</i> .....	0.0004	0.0006	0.0006	0.0007
<i>E. typhosa</i> .....	0.0004	0.0006	0.0006	0.0007
<i>S. conjunctivae</i> .....	0.0006	0.0008	0.0008	0.0010
<i>S. sonnei</i> .....	0.0004	0.0007	0.0006	0.0008
<i>S. dysenteriae</i> .....	0.0004	0.0005	0.0006	0.0007
<i>A. aerogenes</i> .....	0.0004	0.0007	0.0008	0.0008
<i>A. cloacae</i> .....	0.0004	0.0007	0.0008	0.0008
<i>E. coli</i> .....	0.0002	0.0005	0.0004	0.0007
<i>E. communior</i> .....	0.0004	0.0007	0.0006	0.0008
<i>E. acidilactia</i> .....	0.0004	0.0006	0.0006	0.0008
<i>P. aeruginosa</i> .....	0.0002	*	0.0004	0.0005
<i>P. ovalis</i> .....	0.0002	0.0005	0.0004	0.0006
<i>P. griseolens</i> .....	0.0001	*	0.0002	0.0005
<i>P. syncyanea</i> .....	0.0002	*	0.0004	0.0005
<i>P. mucedolens</i> .....	0.0002	*	0.0004	0.0005
<i>S. marcescens</i> .....	0.0006	0.0007	0.0008	0.0010
<i>R. agilis</i> .....	0.0001	0.0005	0.0004	0.0006
<i>R. rosaceus</i> .....	0.0001	0.0005	0.0004	0.0006
<i>Torula rosea</i> .....	0.0020	0.0011	†	0.0080
<i>F. saureolens</i> .....	0.0006	0.0007	0.0008	0.0008
<i>A. lipolyticum</i> .....	0.0006	0.0007	0.0008	0.0010
<i>B. subtilis</i> .....	0.0006	0.0008	0.0008	0.0010
<i>B. mesentericus</i> .....	0.0004	0.0007	0.0006	0.0008
<i>B. mycoides</i> .....	0.0004	0.0007	0.0006	0.0008
<i>B. fusiformis</i> .....	0.0002	0.0005	0.0004	0.0007
<i>B. metiens</i> .....	0.0004	0.0008	0.0006	0.0010
<i>C. intermedium</i> .....	0.0004	0.0007	0.0006	0.0008
<i>A. faecalis</i> .....	0.0004	0.0007	0.0006	0.0008
<i>P. vulgaris</i> x 19.....	0.0004	0.0007	0.0006	0.0008
<i>S. aureus</i> .....	0.0004	0.0007	0.0008	0.0008
<i>S. candidus</i> .....	0.0002	0.0007	0.0006	0.0008
<i>S. albus</i> .....	0.0002	0.0005	0.0006	0.0007
<i>S. flava</i> .....	0.0002	0.0005	0.0004	0.0006
<i>Sarcina lutea</i> .....	0.0002	0.0005	0.0004	0.0007

\* No growth in lowest concentration employed.

† Growth in highest concentration employed.

0.4 M depressed slightly or had no effect on the toxicity of cerium chloride and increased slightly or had no effect on the toxicity of cerium sulfate. Sodium sulfate in 0.1 M concentration markedly reduced the toxicity of cerium sulfate (table 3), but was without significant effect when used with cerium chloride.



Magnesium chloride (0.5 M) generally diminished the toxicity of cerium chloride, but magnesium sulfate (0.5 M) was without effect. The chlorides (0.5 M) of calcium and barium slightly increased the toxicity of cerium chloride, whereas barium sulfate, lithium chloride, ammonium sulfate, and ammonium chloride were without significant effect.

*The toxicity of cerium chloride for fungi.* In all the preceding experiments it was observed that the *Torula* culture was far more tolerant of the cerium compounds than were the bacteria. In order to determine whether other common fungi are equally tolerant of cerium, a yeast extract glucose peptone medium was prepared with concentrations of cerium chloride sufficient to inhibit all the bacteria employed in this study. Thirty-five strains of fungi were inoculated on the media by streaking, and the results were read after incubation for 2 days at room temperature.

The following organisms were employed: *Debaromyces tyrocola*, *Endomyces hordei*, *Monilia krusei*, *Mycoderma valida*, *Pichia farinosus*, *Saccharomyces cerevisiae* Froberg, *Saccharomyces* of Curtis, *Saccharomyces cerevisiae* Saaz, *Schizosaccharomyces mellacei*, *Torula* "Hansen" sp., *Torula humicola*, *Torula mucilaginoso*, *Torula spherica*, *Torula datilla*, *Torula colliculosa*, *Torula sanguinea*, *Torula* "pink" sp., *Torula fructicola*, *Torula liconde*, *Torula fermentati*, *Torula kefyi*, *Torula lactosa*, *Torula candida*, *Zygosaccharomyces priorianus*, *Zygosaccharomyces chevalieri*, *Vermicularia* sp., *Fusarium* sp., *Phytophthora* sp., *Neocosmospora* sp., *Dothiorella* sp., *Cunninghamella* sp., *Trichoderma* sp., *Pythium* sp., *Rhizopus* sp., and *Aspergillus* sp.

All the fungus cultures grew as well in the presence of 0.0014 M cerium chloride, the highest concentration employed, as in the control medium.

*The bacteriostatic activity of lanthanum and thallium.* The toxicity of lanthanum chloride and thallium nitrate for the selected bacteria was determined by the same methods employed in the preceding experiments. The results are presented in table 4. The order of toxicity of lanthanum and thallium was found to be approximately the same as that of cerium. Again, some species were observed to be relatively more resistant than others. The organisms most tolerant of the salts were found to be certain species of *Bacillus*, *Serratia marcescens*, *Sarcina conjunctivae*, *Achromobacter lipolyticum*, and *Torula rosea*. The most susceptible organisms were species of the genus *Pseudomonas*.

#### CONCLUSIONS

The salts of cerium, lanthanum, and thallium were found to be definitely more toxic for the bacteria than for the fungi included in this study.

The 39 species of bacteria were prevented from growth in concentrations of cerium chloride varying from 0.0006 to 0.0012 M; in cerium nitrate from 0.0004 to 0.0008 M; in cerium sulfate from 0.0006 to 0.0008 M; in lanthanum chloride from 0.0002 to 0.0008 M; and in thallium nitrate from 0.0005 to 0.0010 M.

The toxicity of cerium sulfate for most bacteria was reduced by the addition of sodium sulfate (0.05 M) to the medium, and the toxicity of cerium chloride was generally decreased by the addition of magnesium chloride (0.05 M). The addition of other salts had little effect on the toxicity of cerium.

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# STUDIES ON THE QUANTITATIVE DIFFERENTIAL ANALYSIS OF MIXTURES OF SEVERAL ESSENTIALLY PURE PENICILLIN TYPES

RICHARD DONOVICK, DANIEL LAPEDES, AND FELIX PANSY

*Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, New Jersey*

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Since the work of Schmidt, Ward, and Coghill (1945) on a method for differentiating various types of penicillins by means of two assay organisms, very little further work has been published in this connection. That various organisms respond to different penicillin types differently is well known (Veldee *et al.*, 1945; Welch *et al.*, 1944; Libby and Holmberg, 1945; Eagle, 1946; Eagle and Musselman, 1946; etc.), but few specific attempts have been made to develop a quantitative test for specific penicillin types based on differential response of the organisms studied. A relatively rough *in vivo* differential assay procedure was recently proposed by Buck, Farr, and Schnitzer (1946) in which *Borreli*a infections in mice were used.

With the recognition of the various penicillin types have come improved methods for separating mixtures of penicillins into the pure components (Fischbach *et al.*, 1946; Craig *et al.*, 1946). As these purification methods have been increasingly used, the need for accurate differential biological assay procedures has been felt more and more. The recent publication of Higuchi and Peterson (1947) presents a procedure with which they attempted to fill this need.

The method described by these authors employs three test organisms: *Staphylococcus aureus* 209-P, *Bacillus brevis*, and "organism E." Using a turbidimetric test, they reported that it was possible by their method to estimate with fair accuracy the composition of mixtures consisting of penicillins G, K, and X. They noted that the assay procedure was based on several assumptions, one of which was that "the effects of penicillins in mixtures on assay organisms are additive."

Since systematic studies on this latter question were under way in this laboratory with *Staphylococcus aureus* Heatley, the work was augmented after obtaining cultures of *B. brevis* and "organism E" through the kindness of Dr. Peterson. The present paper includes studies on the effects of known mixtures of several penicillin types on these three species of organisms.

A cursory survey of the literature leads one to believe that activities assigned to each of the various penicillin types actually have been established in some cases with mixtures of several types of penicillin. For example, penicillin F, has been reported to have activities of 1,440 to 1,490 units per mg (Schmidt, Ward, and Coghill, 1945) and 1,550 units per mg (Higuchi and Peterson, 1947). Values of 845 to 935 units per mg (Schmidt *et al.*, 1945), 850 (Coghill and Koch, 1945), 900 (Welch *et al.*, 1944), and 1,000 units per mg (Libby and Holmberg,

1945) have been assigned to penicillin X. The slight shift in the assigned activity of penicillin G from 1,650 units per mg (Welch *et al.*, 1944) to 1,667 units per mg (Veldee *et al.*, 1945) with consequent slight changes in the relative activities of the other penicillin types cannot account for these discrepancies. The various activities reported may be attributable to strains of organisms used for assay, to the assay procedures themselves, or to the varying degrees of purity of the preparations used.

Since the chief purpose of the present work was to study the effect, if any, of one penicillin type on the action of another, efforts were made to use only penicillin preparations the purity of which were as thoroughly established as possible.

#### PENICILLIN PREPARATIONS USED

*Penicillin G*<sup>1</sup> (Cra A-328-36). This is a crystalline sodium salt prepared from commercial penicillin by chromatography and recrystallized several times. The chemical analysis agreed well with theoretical:

Found: C, 53.81; H, 4.85.

Calc.: C, 53.92; H, 4.81.

Craig countercurrent distribution studies of this preparation revealed that 90 per cent (by weight, 93 per cent of the activity) was contained in the main band, indicating it to be an essentially homogeneous material. The remaining 10 per cent consisted of inactive impurities, possibly inactivation products formed during the distribution experiment.

Bioassays with *Staphylococcus aureus* Heatley against previously well-established standards showed its activity to agree very well with the defined activity of penicillin G, i.e., 1,667 units per mg, suggesting a purity at least as good as any of the materials used in establishing such standards.

*Penicillin K*<sup>1</sup> (AV-73). This is a crystalline ammonium salt obtained by partition chromatography. The chemical analysis agreed well with the theoretical:

Found: C, 53.34, 53.10; H, 8.05, 8.12; N, 11.62; S, 8.92; Moisture, (H<sub>2</sub>O) 3.46.

Calc: C, 53.46; H, 8.13; N, 11.69; S, 8.99.

As will be shown later, careful bioassay of this preparation gave an activity of 2,540 units per mg, which is about 10 per cent higher than the figure of 2,300 usually assigned to penicillin K (Coghill and Koch, 1945). The subtilis:staphylococcus ratio of 0.36 was in good agreement with that reported to be characteristic of penicillin K (Coghill and Koch, 1945).

*Penicillin X* (NRRL-1717-39A). The penicillin X used for these studies was supplied us through the kindness of Dr. F. H. Stodola, of the Northern

<sup>1</sup> The authors are indebted to Drs. O. P. Wintersteiner and M. Adler of the Division of Organic Chemistry of the Squibb Institute for Medical Research for the penicillins G and K used in these studies as well as the chemical and physical data describing these preparations.

Regional Research Laboratory, who described it as an analytically pure preparation having the following analysis:

Calc. for  $C_{16}H_{17}N_2O_5S$  Na: C, 51.6; H, 4.60.

Found: C, 51.8; H, 4.89.

Their bioassays with *Staphylococcus aureus* (strain not specified) indicated an activity of 920 units per mg.

#### ASSAY PROCEDURE

Higuchi and Peterson (1947) in their differential assay procedure plotted turbidimetric readings of growth against units of penicillin per ml. When large numbers of assays are involved, a technique similar to that used for streptomycin assay (Donovick *et al.*, 1945) has proved preferable in our hands. It is perhaps true that readings of partial inhibition, as were done by Higuchi and Peterson (1947), may be more accurate when the curves, obtained by plotting turbidimeter readings against units of penicillin per ml, are not steep, which was the case with their strain of *Staphylococcus aureus*. On the other hand, for organisms such as *B. brevis* and "organism E," the curves were very steep, showing a change from little inhibition to almost complete inhibition over a very narrow range of penicillin concentrations. Hence, it appeared to us that little could be gained through the use of a turbidimeter for reading end points. The three test organisms used for the present work were *Staphylococcus aureus* Heatley and, as already indicated, two species used by Higuchi and Peterson (1947), viz., *B. brevis* and "organism E."

Sixteen-hour cultures of the three organisms were diluted as follows: *Staphylococcus aureus* Heatley,  $1 \times 10^{-6}$  in yeast beef broth (Difco); *B. brevis*,<sup>2</sup>  $0.25 \times 10^{-5}$  in "Peterson B"<sup>3</sup> broth; and "organism E",  $1 \times 10^{-5}$  in "Peterson E"<sup>3</sup> broth. These dilutions gave counts of approximately 1,000 organisms per ml.

Two-ml volumes of inoculated broths were dispensed with sterile automatic syringes into sterile tubes measuring 13 by 100 mm. The penicillin solution to be assayed, appropriately diluted, was then added to the 2-ml volumes of inoculated broth by means of acid-cleaned, sterile, 0.2-ml Kahn pipettes in the following amounts: 0.10, 0.088, 0.077, 0.068, 0.059, 0.052, 0.046, 0.040, 0.035,

<sup>2</sup> It was found to be advisable to grow *B. brevis* in a shallow layer of broth to obtain sufficiently heavy growth in 16 hours to allow the indicated dilution for the tests.

<sup>3</sup> These media were used by Dr. Peterson in some of his early work and were recommended to us by him (personal communication). They had the following compositions:

	Peterson "B" broth g/liter	Peterson "E" broth g/liter
Peptone.....	6.0	6.0
Yeast extract (Difco).....	3.0	3.0
Glucose.....	1.0	2.0
K <sub>2</sub> HPO <sub>4</sub> .....	3.2	0.5
KH <sub>2</sub> PO <sub>4</sub> .....	2.0	5.0
pH.....	6.8	6.0

and 0.030 ml. The racks containing the tubes of inoculated broth were kept at 5 C prior to the addition of penicillin. Three racks at a time (i.e., one rack of each of the three test organisms) were removed from the icebox, the penicillin was added, and the racks were returned at once to the cold room (5 C). When penicillin had been added to all the racks for a given day, they were all placed in the appropriate incubators<sup>4</sup> at one time and incubated for 15½ to 16½ hours.

The tests, after the tubes were vigorously shaken, were read under a fluorescent day lamp. Absence of growth was recorded as (—), an intermediate degree of growth as (±), and almost complete or complete growth as (+). The end point was considered to be the last (—) in a (—) (+) series; and the mid-point between (—) and (±) in a (—) (±) (+) series. Since in the present investigations the concentrations (by weight) of penicillin in the solutions tested were known, the minimal inhibiting concentrations (M.I.C.) were readily calculated from the volume of penicillin solution added to the end point tube.

Early in the present studies aqueous solutions of each type of penicillin were prepared from carefully weighed samples. The desired mixtures were made by mixing appropriate proportions of the various solutions. All samples were then dispensed in acid-cleaned, sterile ampoules, in ca. 1-ml amounts, and the ampoules sealed and frozen in a CO<sub>2</sub>-alcohol bath. The ampoules were then stored in a CO<sub>2</sub> box until used. When assays were to be made, enough ampoules for that days' work were thawed, and the contents were diluted with distilled water and assayed.

It will be noted that Higuchi and Peterson (1947) expressed penicillin concentrations in terms of the standard unit, "in order to compare the results obtained . . . with previous results." As a consequence, the algebraic expressions which they derived for calculating compositions of penicillin mixtures yielded results in units per cent. The present authors feel that where three test organisms and three or more types of penicillin are involved the use of units leads to confusion and obscures various relationships. This will be discussed more fully below, but suffice it to say for the moment that all M.I.C. data were gathered and are here reported in terms of actual weights of penicillin per unit volume, and the equations given below yield results in percentage by weight. It is obvious that the composition of a mixture containing, e.g., 50 per cent G and 50 per cent K by weight is quite different from one the activity of which consists of 50 units of G and 50 units of K.

Tests on known mixtures of two or more types of penicillin were always accompanied by controls consisting of tests on solutions containing separately the individual components involved in the mixtures. Hence, large numbers of assays of the solutions containing only single types of penicillin were conducted. In table 1 are shown the results of the tests on these control solutions.

Comparison of the M.I.C. values shown in table 1 with those given by Higuchi and Peterson (1947) reveals surprising differences in findings. The cause of these differences is uncertain, but several explanations suggest themselves.

<sup>4</sup> *Staphylococcus aureus* and *B. brevis* were incubated at 37 C; "organism E" at 45 C.

TABLE 1

*Minimal inhibiting concentrations of penicillin in terms of weight*

PENICILLIN	EXPERIMENT NO.	M.I.C.		
		<i>S. aureus</i> (Heatley)	<i>B. brevis</i>	"Organism E"
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
G (CrA-328-36)	1	0.00790 (27)*	0.0125 (25)	0.0311 (23)
G (CrA-328-36)	2	0.00770 (126)	0.0150 (120)	0.0308 (120)
G (CrA-328-36)	3	0.00720 (65)	0.0145 (60)	0.0329 (55)
Average G.....		$0.00755 \pm 0.735\%$ †	$0.0146 \pm 0.957\%$	$0.0317 \pm 0.897\%$
K (AV-73)	1	0.00463 (8)	0.0458 (8)	0.0686 (8)
K (AV-73)	2	0.00515 (31)	0.0475 (28)	0.0690 (29)
K (AV-73)	3	0.00480 (24)	0.0458 (26)	0.0641 (25)
Average K.....		$0.00495 \pm 1.29\%$	$0.0465 \pm 1.17\%$	$0.0670 \pm 2.14\%$
X (NRRL-1717-39A)	1	0.0136 (51)	0.0535 (49)	0.0283 (43)
X (NRRL-1717-39A)	2	0.0133 (26)	0.0535 (22)	0.0277 (24)
X (NRRL-1717-39A)	3	0.0143 (24)	0.0585 (24)	0.0289 (24)
X (NRRL-1717-39A)	4	0.0154 (30)	0.0550 (29)	0.0342 (31)
Average X.....		$0.0140 \pm 1.04\%$	$0.0545 \pm 1.10\%$	$0.0295 \pm 1.49\%$

\* Figure in parenthesis represents number of assays conducted on the specific sample.

† The standard errors shown were calculated on the results of the total number of assays carried out with a specific preparation. The authors are indebted to Mr. Ross Blue of E. R. Squibb &amp; Sons for this statistical analysis.

It is possible that slight differences in media, or perhaps in variations occurring in the cultures, between the time Higuchi and Peterson conducted their tests and the time we received these cultures may have accounted in part for these differences. Probably even more important was that in the present work complete inhibition was taken as the end point, whereas some point of partial inhibition (but which is not clearly indicated) was used as the end point by Higuchi and Peterson (1947).

The latter authors reported M.I.C. values, for their strain of *Staphylococcus aureus* (209-P), which are close to twice as great as those found for *Staphylococcus aureus* Heatley in the present work. Though the strains of *B. brevis* and "organism E" used for these studies were the same as those used by the foregoing authors, the minimal inhibiting concentrations reported by them for the various penicillin types studied are quite different from those reported here. It is interesting, therefore, to note how the results compare when the present data are converted into units.

Assigning to penicillin G its defined activity of 1,667 units per mg, the K penicillin used in the present work would have an activity of  $1,667 \times \frac{7.55}{4.95} = 2,540$  units per mg, and the X used would have  $1,667 \times \frac{7.55}{14.0} = 898$  units per

TABLE 2  
Minimal inhibiting concentrations of penicillin in terms of units

TEST ORGANISM	PENICILLIN TYPE	ACTIVITY OF PENICILLIN UNITS PER MG	M.I.C.		RATIO OF M.I.C.'S IN TERMS OF UNITS	
			$\mu\text{g}$ per liter	Units per liter	G/K	G/X
<i>S. aureus</i> (Heatley)	G	1,667	7.55	12.6	1	1
<i>S. aureus</i> (Heatley)	K	2,540	4.95	12.6		
<i>S. aureus</i> (Heatley)	X	898	14.0	12.6		
<i>B. brevis</i>	G	1,667	14.6	24.3	0.206	0.497
<i>B. brevis</i>	K	2,540	46.5	118.1		
<i>B. brevis</i>	X	898	54.5	48.9		
"Organism E"	G	1,667	31.7	52.8	0.310	1.97
"Organism E"	K	2,540	67.0	170.1		
"Organism E"	X	898	29.5	26.5		

mg. On the basis of these potencies, conversion of the M.I.C. values shown (in terms of weight) in table 1 to M.I.C. in terms of units give the results shown in table 2.

Thus, despite the differences between the absolute (weight) M.I.C. values reported here and by Higuchi and Peterson (1947), equations very similar to those of the latter authors, based on relative (unitage) M.I.C. ratios, may be set up.

#### STUDIES WITH KNOWN MIXTURES CONTAINING TWO TYPES OF PENICILLIN

To determine whether the effects of the various types of penicillins were truly additive, mixtures containing two types were first studied. The findings with such two component mixtures are shown in tables 3, 4, and 5. Studies were then undertaken with three component mixtures and these results are listed in table 6. The values listed under the columns headed "theor." were calculated by means of equations (1), (2), and (3) below after substituting in the known values of a, b, and c. These equations hold true only in so far as the effects of the penicillins are additive, in which case there would be a direct proportionality between the composition of a given mixture and the M.I.C. values of this mixture for the three test organisms.

The following equations relate the concentration (in terms of weight per volume) of mixed penicillins at the end point to the composition of the mixture:

In a mixture of penicillins, let—

a = per cent, by weight, of penicillin G

b = per cent, by weight, of penicillin K

c = per cent, by weight, of penicillin X

MsG = M.I.C. of pure penicillin G for *Staphylococcus aureus*

MsK = M.I.C. of pure penicillin K for *Staphylococcus aureus*

MsX = M.I.C. of pure penicillin X for *Staphylococcus aureus*



Similarly let—

MbG = M.I.C. of pure penicillin G for *Bacillus brevis*

MeG = M.I.C. of pure penicillin G for "organism E," etc.

Ms = M.I.C. of mixture of penicillin for *Staphylococcus aureus*

Mb = M.I.C. of mixture of penicillin for *Bacillus brevis*, etc.

TABLE 3

*Minimal inhibiting concentrations of known mixtures of penicillins G and K*

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms µg per liter			Mb µg per liter			Me µg per liter		
Per cent G	Per cent K	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.
100	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
90	10	7.16	7.50	1.05	15.7	15.5	0.99	33.4	35.5	1.06
80	20	6.83	7.00	1.02	16.9	17.5	1.03	35.4	37.5	1.06
65	35	6.38	7.18	<i>1.12</i>	19.2	20.0	1.04	38.9	44.3	<i>1.14</i>
50	50	5.98	7.01	<i>1.17</i>	22.2	22.0	0.99	43.1	49.6	<i>1.15</i>
35	65	5.63	6.75	<i>1.20</i>	26.4	28.5	1.08	48.3	54.6	<i>1.13</i>
20	80	5.31	6.34	<i>1.19</i>	32.4	33.5	1.03	54.7	55.0	1.00
10	90	5.12	5.50	1.07	38.2	39.0	1.02	60.4	65.0	1.08
0	100	4.95†	4.95	1.00	46.5†	46.5	1.00	67.0†	67.0	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

\* Composition in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

TABLE 4

*Minimal inhibiting concentrations of known mixtures of penicillins G and X*

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms µg per liter			Mb µg per liter			Me µg per liter		
Per cent G	Per cent X	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.
100	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
90	10	7.92	8.82	<i>1.11</i>	15.8	17.0	1.08	31.4	35.4	<i>1.13</i>
80	20	8.32	8.87	<i>1.06</i>	17.1	17.5	1.02	31.2	34.0	<i>1.09</i>
65	35	9.00	9.00	1.00	19.6	20.5	1.02	30.8	33.5	1.09
50	50	9.84	10.5	1.07	23.0	22.5	0.98	30.5	32.5	1.06
35	65	10.8	11.5	<i>1.07</i>	28.8	27.0	0.94	30.2	31.6	1.04
20	80	12.0	13.3	<i>1.11</i>	35.2	36.0	1.02	29.9	31.9	<i>1.06</i>
10	90	12.9	13.9	<i>1.08</i>	42.7	43.0	1.01	29.7	32.5	<i>1.09</i>
0	100	14.0†	14.0	1.00	54.5†	54.5	1.00	29.5†	29.5	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

\* Composition in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

Then,<sup>5</sup>

$$(1) \quad M_s = \frac{100}{\frac{a}{M_sG} + \frac{b}{M_sK} + \frac{c}{M_sX}} = \frac{100}{\frac{a}{7.55} + \frac{b}{4.95} + \frac{c}{14.0}}$$

$$(2) \quad M_b = \frac{100}{\frac{a}{M_bG} + \frac{b}{M_bK} + \frac{c}{M_bX}} = \frac{100}{\frac{a}{14.6} + \frac{b}{46.5} + \frac{c}{54.5}}$$

$$(3) \quad M_e = \frac{100}{\frac{a}{M_eG} + \frac{b}{M_eK} + \frac{c}{M_eX}} = \frac{100}{\frac{a}{31.7} + \frac{b}{67.0} + \frac{c}{29.5}}$$

$$(4) \quad a + b + c = 100.$$

TABLE 5

*Minimal inhibiting concentrations of known mixtures of penicillin K and X*

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms µg per liter			Mb µg per liter			Me µg per liter		
Per cent K	Per cent X	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.
100	0	4.95†	4.95	1.00	46.5†	46.5	1.00	67.0†	67.0	1.00
90	10	5.28	5.50	1.04	47.2	47.3	1.00	59.5	62.5	1.05
80	20	5.69	5.44	0.96	47.8	54.1	<i>1.13</i>	53.5	49.0	0.92
65	35	6.41	6.50	1.01	49.0	56.6	<i>1.15</i>	46.5	45.5	0.98
50	50	7.38	8.15	<i>1.10</i>	50.2	56.7	<i>1.13</i>	41.0	47.8	<i>1.16</i>
35	65	8.54	9.00	1.05	51.4	55.0	1.07	36.8	38.6	1.02
20	80	10.3	11.0	1.07	52.6	54.3	1.03	33.2	32.2	0.97
10	90	11.9	12.5	1.05	53.6	56.5	1.05	31.2	33.0	1.06
0	100	14.0†	14.0	1.00	54.5†	54.5	1.00	29.5†	29.5	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

\* Compositions in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

The ratio between the "theoretical" M.I.C. and the experimentally determined M.I.C. is a measure of the extent to which the effects of the various penicillin types are additive since, as already indicated, the equations above are based on the assumption that they are additive. Hence, the "theoretical" value and the experimentally determined value for a given mixture should be equal, within experimental error, when the effects are additive.

It was found that with certain two component mixtures, the experimental M.I.C. values were as high as 20 per cent greater than expected. This would

<sup>5</sup> For convenience in handling the figures, the M.I.C. values are here given in terms of µg per liter.

indicate that a certain amount of interference with the action of one penicillin is caused by the presence of a second penicillin.

It may well be asked whether these deviations from the theoretical M.I.C.'s are significant. Handicaps in biological assay procedures at best are the relatively large standard deviations which occur unless a large number of assays are done. Consequently, in order to determine whether these observed deviations were statistically significant, a great many of the data involved were subjected to statistical analysis.<sup>6</sup> The findings of this analysis are shown in figure 1 and indicate that the deviations are significant.<sup>7</sup> In two component mixtures consisting of penicillins G and K, significantly larger amounts of penicillin were

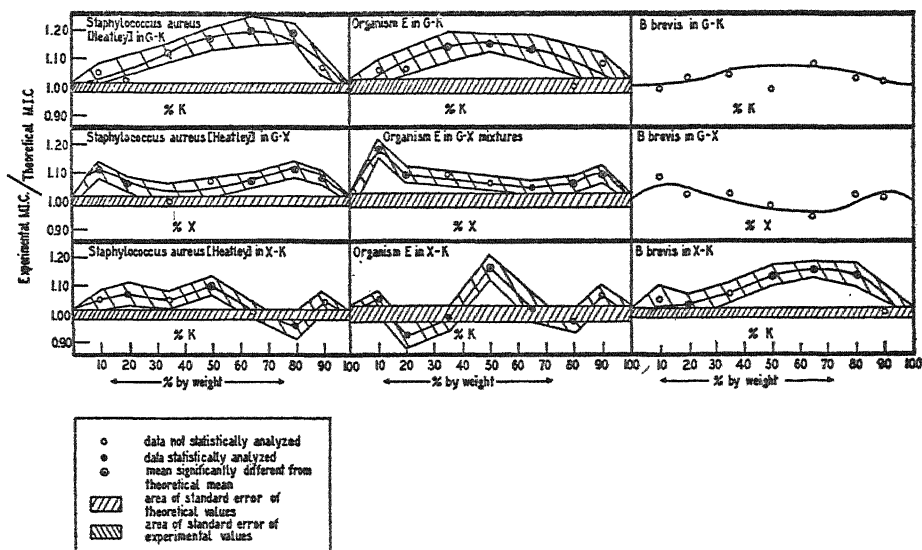


FIG. 1. DEVIATION OF EXPERIMENTAL M.I.C. VALUES FROM THEORETICAL VALUES IN TWO-COMPONENT MIXTURES OF PENICILLIN

required to inhibit *Staphylococcus aureus* Heatley and "organism E" than would be expected on the basis of the M.I.C. values of the individual penicillins. This is especially evident in mixtures containing between 35 and 65 per cent K. Although the deviation from theory for such mixtures in the case of *B. brevis*

<sup>6</sup> The authors are indebted to Mr. Ross Blue of the Division of Product Control of the Chemical and Biological Laboratories, E. R. Squibb & Sons, for carrying out this task.

<sup>7</sup> In accordance with common practice, the difference between a given mean experimental M.I.C. value and the corresponding theoretical value was considered significant when this difference was at least two times as great as the standard error of the difference. In the cases considered as significant in figure 1, the differences between experimental and theoretical M.I.C. values were from 2.1 to 5.1 times as great as the standard errors of the differences. (For a complete discussion on such statistical procedures, see such standard texts as F. C. Mills, *Statistical Methods*, Henry Holt and Co., New York, 1939.)

is apparently not statistically significant, yet the tendency appears to be in the same direction.

When the mixture consisted of penicillins G and X, deviations reached maxima in two regions, one in the vicinity of 10 per cent X and another at 80 to 90 per cent X for both *Staphylococcus aureus* Heatley and "organism E." The picture appeared to be similar here in the case of *B. brevis*, but again the deviations from theory were not statistically significant.

The data on staphylococcus and "organism E" in K-X mixtures were very inconclusive except in the vicinity of 50-50 mixtures in which the amount of penicillin required to inhibit was again significantly greater than expected. In this case the data on *B. brevis* were quite clear-cut. Significantly more penicillin was required to inhibit this organism than would be expected in mixtures covering the range of 50 to 80 per cent X.

It is of interest to note that in none of the cases studied was the experimental M.I.C. significantly less than the theoretical figure. It would appear, therefore, that in two component mixtures of penicillins, one penicillin may interfere with the action of the other, thereby requiring a greater total amount of penicillin to cause inhibition than might be expected. Since very little is understood of the mode of action of the penicillins, it is not possible at present to explain this apparent interference. It is not even clear whether these compounds act within the bacterial cell or upon the cell surfaces, nor, in fact, whether all the penicillins inhibit growth in identically the same fashion.

If, as a working hypothesis, one were to assume that the penicillins act within the cell rather than upon the surface, then one might tentatively propose that the apparent interference may actually be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell. This would result in the composition of the penicillin mixture inside the cell being different from that outside. For example, it can be seen in table 3 that for *Staphylococcus aureus* Heatley the experimental M.I.C. of a mixture containing 20 per cent G and 80 per cent K is equal to the theoretical M.I.C. of a 65 per cent G and 35 per cent K mixture. On the other hand, for "organism E" the experimental M.I.C. of a mixture containing 35 per cent G and 65 per cent K is equal to the theoretical M.I.C. of a 20 per cent G and 80 per cent K mixture. If differential adsorption is in fact the reason for the observed interference, then one might expect penicillin K to be adsorbed more readily than penicillin G by *Staphylococcus aureus* Heatley and the reverse to be true for "organism E." Studies on adsorption of penicillin by bacteria which are under way in this laboratory (Rake *et al.*, to be published) may perhaps lend weight for or against such a hypothesis.

Of course, the interference may be due to competition at a site of action of penicillin within the bacterial cell, but little can be said about this in the present state of knowledge of the mode of action of the penicillins.

#### STUDIES WITH MIXTURES CONTAINING THREE TYPES OF PENICILLIN

The question of deviation of behavior from the expected becomes increasingly difficult to answer with the increase in the number of penicillins involved. It

was assumed, for purposes of calculation, that the effects of the penicillins were additive, and equations were derived expressing the relationship between the M.I.C. of a mixture and its composition by weight—equations (1), (2), (3), and (4). Using these equations the “theoretical” values of Ms, Mb, and Me for 10 three-component mixtures were calculated, and at the same time these values were determined experimentally for these mixtures. The comparison of these two sets of data is shown in table 6.

It will be noted that with *Staphylococcus aureus* and *B. brevis* the ratios between “theoretical” and experimental M.I.C. values were usually very close to 1.0, whereas with “organism E” in 5 out of 10 mixtures the ratios indicated

TABLE 6  
Minimal inhibiting concentrations of known mixtures of penicillins G, K, and X

COMPOSITION* OF MIXTURE			S. AUREUS			B. BREVIS			“ORGANISM E”		
			Ms µg per liter			Mb µg per liter			Me µg per liter		
Per cent G	Per cent K	Per cent X	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.	Theor.	Found	Found Theor.
100	0	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
0	100	0	4.95	4.95	1.00	46.5	46.5	1.00	67.0	67.0	1.00
0	0	100	14.0	14.0	1.00	54.5	54.5	1.00	29.5	29.5	1.00
80	10	10	7.50	7.50	1.00	17.0	16.5	0.97	33.2	38.0	1.14
60	20	20	7.45	7.50	1.01	20.4	19.5	0.96	34.9	36.5	1.05
40	40	20	6.75	6.50	0.96	25.2	25.0	0.99	39.5	39.5	1.00
40	20	40	8.19	8.50	1.04	25.6	25.5	1.00	34.4	38.0	1.10
33.3	33.3	33.3	7.39	7.50	1.01	27.7	25.5	0.92	37.3	39.0	1.05
20	60	20	6.17	6.50	1.05	33.0	35.5	1.07	45.5	48.0	1.05
20	40	40	7.36	8.00	1.09	33.8	37.0	1.09	38.8	44.0	1.13
20	20	60	9.11	9.40	1.03	34.5	33.5	0.97	33.7	38.0	1.13
10	10	80	11.0	11.5	1.04	42.2	43.4	1.03	31.5	37.0	1.17
10	80	10	5.49	4.95	0.90	38.6	37.5	0.97	54.0	55.0	1.02

\* Composition in terms of grams of given penicillin per 100 grams total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

that from 10 to 17 per cent more penicillin than expected was required. Although the experimental error in this work was probably no greater than that with the two component mixtures (since the two sets of data were gathered under identical conditions), the error in the “theoretical” figures would be statistically higher, having been derived algebraically from data on each of the three types of penicillin used, and the total error would contain errors from figures on each type of penicillin. Consequently, no attempts were made to establish the degree of significance of these deviations. Instead, attention was turned to the question of how well the compositions of the various mixtures could be calculated from the experimental data.

Going back to equations (1), (2), (3), and (4) it can be seen that, by solution with simultaneous equations, the concentration (per cent by weight of total

penicillin) of each component in any mixture can be expressed in three ways, i.e., in terms of  $M_s$  and  $M_b$ ,  $M_s$  and  $M_e$ , or  $M_b$  and  $M_e$ . Solutions in terms of two of these three combinations are shown in the following equations:

$$(5) \quad a = \frac{2,054}{M_b} - \frac{49.6}{M_s} - 34.16$$

$$(6) \quad b = \frac{789}{M_s} - \frac{961}{M_b} - 38.69$$

$$(7) \quad c = 172.8 - \frac{1,093}{M_b} - \frac{739}{M_s}$$

or

$$(8) \quad a' = \frac{15,320}{M_e} + \frac{2,226}{M_s} - 678.5$$

$$(9) \quad b' = 262.7 - \frac{273.7}{M_s} - \frac{7,169}{M_e}$$

$$(10) \quad c' = 515.7 - \frac{8,155}{M_e} - \frac{1,951}{M_s}$$

Thus theoretically it should be possible to determine the composition of a three-component mixture through the use of two test organisms rather than three.

When the "theoretical" values for  $M_s$ ,  $M_b$ , or  $M_e$ , as the case may be, are substituted into the foregoing equations, the correct values for  $a$ ,  $b$ , and  $c$  (or  $a'$ ,  $b'$ , and  $c'$ ) are of course obtained. In table 7 are shown the results when the experimentally determined M.I.C. values are used in these equations. Considering that the experimental M.I.C. values, even with "organism E," exceeded the "theoretical" value by as much as 15 per cent in only one case, it was at first surprising to find that the use of equations (8), (9), and (10)—which involve the use of  $M_s$  and  $M_e$ —yielded such meaningless results for  $a'$ ,  $b'$ , and  $c'$  (i.e., per cent G, per cent K, per cent X). However, examination of these data with the aid of triangular co-ordinate graph paper (Keuffel and Esser Co., no. 359-32) supplied the explanation for such results.

Equations (1), (2), and (3) may be expressed:

$$(11) \quad a/7.55 + b/4.95 + c/14.0 = 100/M_s$$

$$(12) \quad a/14.6 + b/46.5 + c/54.5 = 100/M_b$$

$$(13) \quad a/31.7 + b/67.0 + c/29.5 = 100/M_e$$

Since  $a + b + c = 100$ , each of these equations may further be expressed in terms of two unknowns instead of three, again through solution with simultaneous equations:

$$(14) \quad 0.0610 a + 0.1305 b = 100/M_s - 7.15$$

$$(15) \quad 0.0470 b + 0.05015 c = 6.85 - 100/M_b$$

$$(16) \quad 0.0166 a + 0.0190 c = 100/M_e - 1.49$$

Each of these equations yields an infinite series of parallel straight lines when plotted on triangular co-ordinate paper, the location and direction of each line depending upon the equation and M.I.C. value (i.e.,  $M_s$ ,  $M_b$ , or  $M_e$ ) involved. With a given three-component mixture of penicillins, having "theoretical" M.I.C. values of, e.g.,  $M_{s1}$ ,  $M_{b1}$ , and  $M_{e1}$ , the composition of the mixture will be given by the location of the point of intersection of the three lines formed by the plotting of equations (14), (15), and (16) into which the given M.I.C. values have been substituted.

TABLE 7

*Compositions of mixtures containing three types of penicillin as calculated from minimal inhibiting concentrations*

KNOWN COMPOSITION* OF MIXTURE			COMPOSITION CALCULATED ON BASIS OF EXPERIMENTAL M.I.C.					
Per cent G	Per cent K	Per cent X	a†	b†	c†	a'‡	b'‡	c'‡
100	0	0	100.0	-0.3	0.1	99.5	0.4	0.1
0	100	0	-0.1	100.1	0	-0.3	100.4	-0.1
0	0	100	-0.1	0.2	100.0	-0.3	0.2	100.0
80	10	10	83.6	8.3	8.1	21.3	38.6	40.3
60	20	20	64.5	17.2	18.2	38.0	29.8	32.3
40	40	20	40.3	44.2	15.9	51.7	39.1	9.3
40	20	40	40.5	16.5	43.1	-13.0	41.9	70.9
33.3	33.3	33.3	39.6	28.9	31.5	11.3	42.4	46.6
20	60	20	15.8	55.7	28.4	-17.0	71.3	45.8
20	40	40	15.1	34.0	50.9	-52.1	65.6	86.8
20	20	60	21.9	16.5	61.9	-38.6	45.0	93.4
10	10	80	8.8	7.8	83.3	-71.0	45.3	125.3
10	80	10	10.6	95.1	-5.3	49.0	78.0	-26.6

\* Composition in terms of grams of given penicillin per 100 grams total penicillin.

† Calculated by use of equations (5), (6), and (7).

‡ Calculated by use of equations (8), (9), and (10).

An example of the use of this graphic procedure is shown in figure 2. For this example a mixture consisting of 40 per cent G, 20 per cent K, and 40 per cent X was used. As was shown in table 6, the "theoretical" M.I.C. values of such a mixture would be  $M_s = 8.19$ ,  $M_b = 25.6$ , and  $M_e = 34.4$   $\mu$ g per liter, respectively. These values were substituted in equations (14), (15), and (16), and the lines drawn after locating for each line two points through which it passed. (In most cases it is simplest to calculate the value of b, when  $a = 0$ , and the value for a, when  $b = 0$ , etc. This locates two points on opposite borders of the graph through which the given line passes.)

In figure 2 the solid lines are those obtained by plotting the equations into which have been substituted the "theoretical" M.I.C. values given above. It can be seen that the three lines intersect at the point when  $a = 40$ ,  $b = 20$ , and  $c = 40$ . Of course, plottings of the three equations will give lines which will intersect at precisely the correct point only so long as the three M.I.C. values are precisely correct. Since the latter are experimentally determined,

such precision cannot be expected. Hence, it is of interest to know how changes in the M.I.C. values will affect the location of the point or points of intersection. To demonstrate this effect each of the three M.I.C. values used in plotting the lines of figure 2 were arbitrarily increased by 10 per cent and new lines plotted corresponding to these new M.I.C. values. This gave the dotted lines shown in figure 2.

It is at once evident that a 10 per cent error in both  $M_s$  and  $M_b$  does not shift the point of intersection of these lines nearly so much as it shifts the point of intersection of the  $M_s$  and  $M_e$  lines. In fact, an error of 10 per cent in the

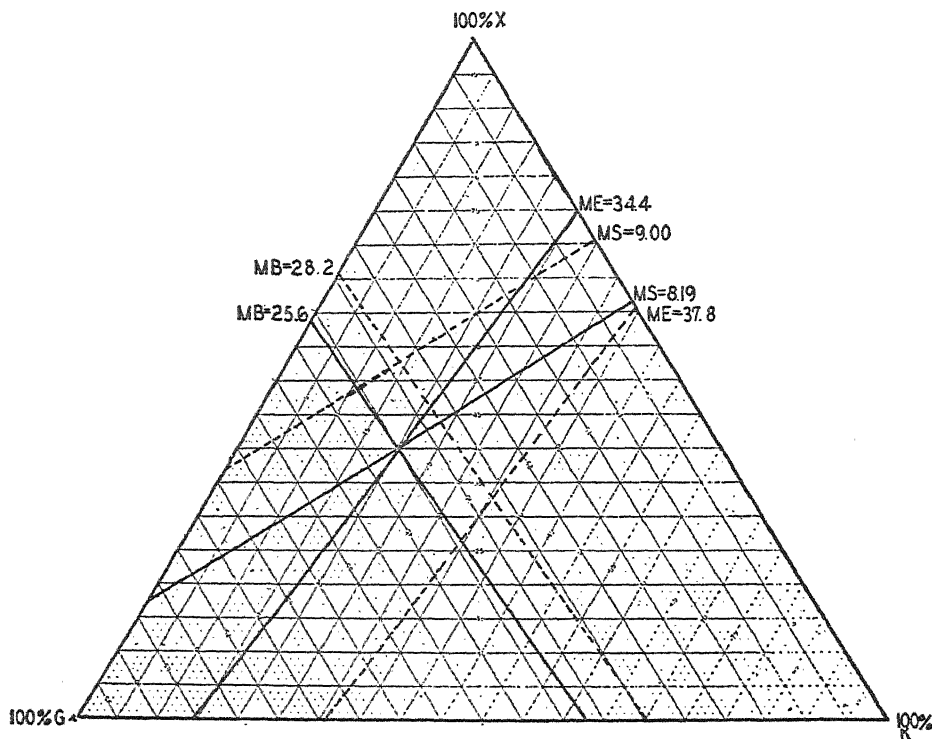


FIG. 2. THE EFFECT OF EXPERIMENTAL ERRORS IN M.I.C. VALUES ON CALCULATED COMPOSITION OF PENICILLIN MIXTURES

$M_e$  value causes its line to intersect the  $M_s$  line somewhere off the graph (at an imaginary point, since in the case demonstrated this would mean a negative value for  $a$ , or less than 0 per cent penicillin G). Examination of this graph indicates that *Staphylococcus aureus* and "organism E" make a poor pair for quantitative differential analysis of penicillin mixtures. *Bacillus brevis* and "organism E" are a somewhat better pair, but the best pair here tested is *Staphylococcus aureus* and *B. brevis*. The graph also indicates why the use of the experimentally determined  $M_s$  and  $M_e$  values (table 6) when used in equations (8), (9), and (10) gave such meaningless values for  $a'$ ,  $b'$ , and  $c'$  (table 7). It



can also be seen why the use of the experimentally determined  $M_s$  and  $M_b$  values gave fairly good figures for  $a$ ,  $b$ , and  $c$  (table 7) when equations (5), (6), and (7) were employed. Thus the use of triangular co-ordinate graph paper in this manner could be of great aid in the search for organisms best suited for quantitative differential analysis of penicillin mixtures.

The 10 three-component mixtures which were studied were analyzed graphically in this manner, calculating the composition from the experimentally determined  $M_s$  and  $M_b$  values. In figure 3 the compositions calculated in this

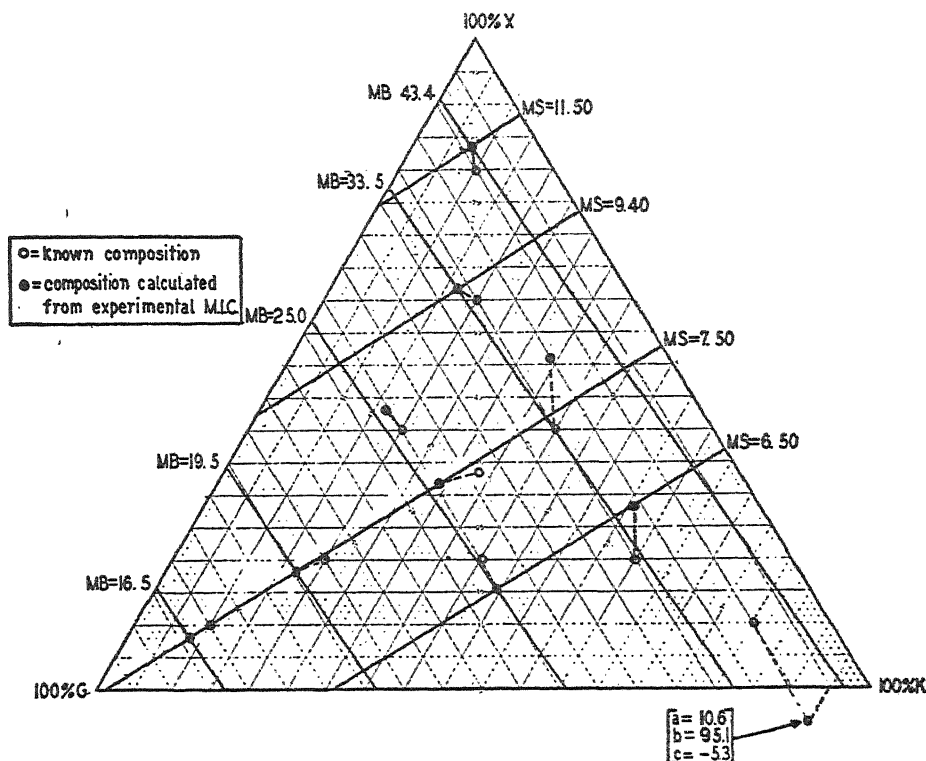


FIG. 3. DIFFERENTIAL ANALYSIS OF PENICILLIN MIXTURES USING *STAPHYLOCOCCUS AUREUS* AND *BACILLUS BREVIS*

manner are shown as solid points, and the known composition of the mixtures are shown as open circles. To simplify the appearance of this graph for purposes of photography some of the  $M_s$  and  $M_b$  lines have been omitted, but their points of intersection (solid dots) are shown.

It will be noted that only when the content of penicillin G fell below ca. 20 per cent of the total penicillin present in a mixture were there marked differences between the estimated and known compositions. Even these differences may have been within experimental error, except for the case of the mixture consisting of 10 per cent G, 80 per cent K, and 10 per cent X. In this case the  $M_s$  value was equal to that for pure K. This resulted in the point, representing

the composition of the mixture, falling outside the limits of the graph. For all mixtures containing more than 20 per cent G, the results were probably all within the range of experimental error.

From the data thus far obtained it has not yet been possible to demonstrate a clear-cut interference of one penicillin with another in three-component mixtures. Hence it is not yet clear what effect the addition of a third penicillin component has on the behavior of an existing two-component system. It appears safe to say, however, that differential penicillin bioassays as they now exist yield only rough approximations of the composition of mixtures even when they consist only of penicillins G, K, and X. The presence of more than three penicillins in a mixture results in even less accuracy in its differential assay results. Greatest value may be derived from such procedures in studying the final purification of a given penicillin species when it is already almost pure, or when considerable chemical data have been gathered on a preparation to show that it consists of no more than three types of penicillin.

It perhaps should be pointed out, in passing, that there are certain real advantages in determining the M.I.C. of relatively pure preparations in terms of weight rather than in units. For example, Hobby *et al.* (1946), in studying several types of penicillin, indicated that the bacterial spectrum of these preparations yielded little information and that only with certain strains of *Escherichia typhosa* was it possible to demonstrate a difference in any of the forms of penicillin. This is true only so long as the activities of the various penicillins are expressed in units, and is so of necessity for *Staphylococcus aureus* by the definition of the unit itself. When the standard unit is used, all penicillin activities are expressed in terms of a measure of the sensitivity of *Staphylococcus aureus* to penicillin G. It can be seen in table 2 that, despite the distinct differences in sensitivity of *Staphylococcus aureus* Heatley to penicillins G, K, and X in terms of weight, these differences are hidden when the M.I.C.'s are given in terms of units. If the M.I.C.'s are given in weights, essentially pure penicillin preparations are readily identified by ratios of M.I.C.'s. Thus the characteristic ratio for penicillin G is:

*Staphylococcus: brevis*: "organism E": = 7.55:14.6:31.7 = 1:1.93:4.2

For penicillin K it is:

$$4.95:46.5:67.0 = 1:9.4:13.5$$

and for penicillin X:

$$14.0:54.5:29.5 = 1:3.9:2.1$$

Or the same data could be presented in another fashion: the K:G M.I.C. ratio for *Staphylococcus aureus* Heatley is 0.655; that of X:G is 1.86. Similarly for *B. brevis*, K:G is 3.18; X:G is 3.73. And for "organism E," K:G is 2.15 and X:G is 0.925. Some studies were also conducted with several penicillin F preparations, but, since there was some doubt as to the purity of the materials available, these data have not been reported here. It appears, however, that

the staphylococcus: brevis: "organism E" ratio of penicillin F is in the vicinity of  $8.4:32:58 = 1:3.8:6.9$ .

In the final purification of preparations of the various penicillin types these triple ratios, as has become the custom to call them, have been of considerable aid as guidelines.

#### SUMMARY

Using *Staphylococcus aureus* Heatley, *Bacillus brevis*, and Peterson's "organism E" as test organisms, it has been shown that in mixtures of two types of penicillin, more penicillin is required to cause inhibition of growth than would be expected from data on the actions of the individual penicillin types. Until the mode (or modes) of action of the penicillins are better understood, this interference on the part of one type of penicillin with the action of another cannot be explained. However, it is tentatively proposed that this phenomenon may be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell.

Equations are given which show the algebraic relationship between the composition of a given penicillin mixture and the weight of total mixed penicillin required to inhibit growth. Through the use of these equations, as well as through the use of a graphic procedure employing triangular co-ordinate paper, it has been shown that only two test organisms are needed for the analysis of mixtures containing three types of penicillin and that *Staphylococcus aureus* Heatley and "organism E" make a poor pair of organisms for such quantitative differential analyses. A better pair of test organisms is that of *Staphylococcus aureus* Heatley and *B. brevis*. However, evidence is also presented to show that even with this pair of organisms relatively slight variations in the experimentally determined minimal inhibiting concentrations cause significant variations in the calculated composition of such mixtures. Hence, such procedures at best give only rough approximations of the composition of penicillin mixtures and are most valuable in the final purification steps of single penicillin types.

The graphic procedure described may prove to be of assistance in finding the best test organisms for such differential analyses.

It has been pointed out that when essentially pure penicillins are involved there are advantages in calculating minimal inhibiting concentrations in terms of weight instead of in units.

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# A BACTERIAL SPRAY APPARATUS USEFUL IN SEARCHING FOR ANTIBIOTIC-PRODUCING MICROORGANISMS

P. G. STANSLY

*Chemotherapy Division, Stamford Research Laboratory, American Cyanamid Company,  
Stamford, Connecticut*

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A number of writers have reviewed the various methods available for isolating antibiotic-producing microorganisms (Waksman, 1945; Pinschmidt and Levy, 1944). These methods have different disadvantages. Some involve individual testing of every organism obtained and are, therefore, extremely laborious. Others, as the bacterial agar plate, appear to be of questionable value (Waksman and Schatz, 1946). Still others, for example, the crowded plate method (Stokes and Woodward, 1942), are objectionable because the less dominant soil organisms tend to be neglected and, in addition, the activity of the observed antibiotic colonies is not directed against a particular test organism but against some chance soil form which happens to be in the vicinity.

The present communication describes a spray apparatus by means of which agar plates containing several or numerous soil colonies, for instance, may be conveniently inoculated with a desired test organism and then reincubated to detect the antibiotic-producing colonies. The advantages of this treatment are apparent. It permits immediate recognition of growth-inhibiting substances specifically directed against the test organism and emanating from antibiotic colonies on plates with numerous inactive organisms. In this way individual testing of vast numbers of organisms present in the heterogeneous soil population, which would ultimately prove to be inactive, is obviated. The many disadvantages attending flooding plates instead of spraying, such as smearing, spreading, and overgrowth of soil colonies which may render recognition and isolation of antibiotic-producing organisms impossible, are largely avoided. The spraying technique has led with a minimum of labor to the isolation of a number of antibiotic-producing organisms including *Bacillus polymyxa*, which produces the antibiotic substance polymyxin (Stansly, Shepherd, and White, in press).

Although the spray apparatus has been designed for the specific purpose given, its application in other problems involving the seeding of agar plates suggests itself.

## THE SPRAY APPARATUS

Although a more elaborate spray apparatus has been constructed for the purpose described, a simple device which has served usefully for several years in this laboratory is shown in figure 1. This apparatus is constructed of readily available materials, requires no special skill in constructing, is simple to operate, and may be sterilized by autoclaving.



# A SIMPLE METHOD FOR CONTROLLED EXPERIMENTATION ON THE PASSAGE OF MICROORGANISMS THROUGH THE DIGESTIVE TRACT OF INSECTS

STANLEY E. WEDBERG AND NORMAN A. CLARKE

*Department of Bacteriology, University of Connecticut, Storrs, Connecticut*

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This paper is a preliminary report on a relatively new technique being employed for controlling the feeding of insects and the collection of their stools for microbiological studies.<sup>1</sup> We are primarily interested in the possible role played by insects in the transmission of disease, particularly enteric diseases, through the agency of food which they may contaminate. To the best of our knowledge no conclusive experiments have been attempted on completely controlled feeding of insects to study their ability to ingest, retain, and to disseminate specific microorganisms. Papers by Steinhaus (1940, 1941) and Gier (1947) suggest a fertile field for investigations of this type.

The latest complete survey on the relationship between microorganisms and insects is probably that presented by Steinhaus (1946), and even this excellent review offers little exact information on this particular phase of the subject. Consequently, our experiments are based upon techniques perfected largely through trial and error. We are indebted, however, to Dr. Lawrence R. Penner of the Zoology Department of the University of Connecticut for his valuable suggestions on the mounting and feeding of the live insects. He has employed similar methods with flies in his studies on poliomyelitis virus.

## MATERIALS AND METHODS

Our experiments are being conducted with *Blaberus craniifer*, a large roach common in Florida. Specimens are shipped to us by air express from Key West. Because of its large size (about 3 to 5 cm long and 2 to 3 cm wide in the adult stage), this roach is particularly well adapted to our feeding studies in which not only the quality but also the quantity of food and organisms ingested can readily be controlled.

By mounting these insects on blocks of paraffin (melting point 51 to 52 C) we have been able to keep them alive in battery jars under completely controlled conditions for weeks at a time. The blocks are first softened by placing them in hot water and then are molded to fit the general contour of the back of the roach. By momentarily flaming the paraffin it can be made to adhere when pressed to the back of an insect which has previously been placed in the freezing compartment of a refrigerator for about five minutes. This chilling procedure tends to keep the roach relatively inactive during the mounting operation. Once contact of the wings is established with the paraffin block, a firmer mount is prepared by embedding the edge of the wings with melted paraffin directed

<sup>1</sup> Frings (1946) has discussed the history of the use of similar techniques by various authors for a number of purposes.

to the area with an eye dropper. The insects are then placed on their backs and the blocks are attached to glass rods for mounting as is shown in figure 1.

Our early attempts to collect stool specimens met with failure when we tried to suspend the insects in an upright position. Too often the roaches regurgitated their food, and cultures made from the stools were contaminated with this regurgitated material. By mounting the subjects on their backs and by trimming the wings at the posterior end, it was possible to collect the stools in a satisfactory

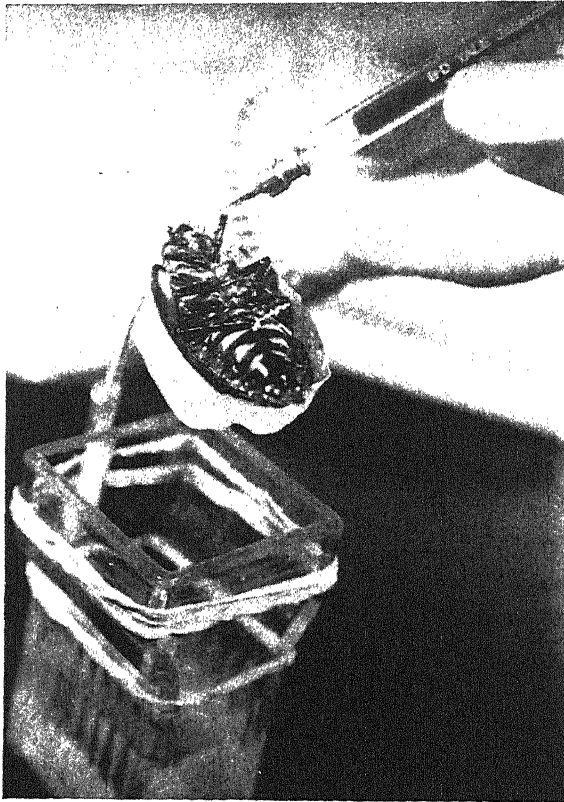


FIG. 1. THE METHOD OF FEEDING THE INSECT

manner. The comfort of the roaches when lying on their backs appears to be optimum under the conditions of the test.

Stool specimens were collected directly on agar plates or on differential media placed below the insect, and these media kept the stools from drying out. A high humidity, one of the apparent prerequisites for keeping these roaches alive for any length of time, was maintained by placing a small amount of water in the bottom of the battery jar (figure 2). By covering the top of the jar with multiple layers of cheesecloth, contamination of the agar plates was minimized while access of air was unimpaired.



Hand feeding of sterile molar solutions of sucrose containing a trace of Difco yeast extract was accomplished with a tuberculin syringe fitted with a 20-gauge needle. Any pure cultures of organisms to be fed were merely added to this sterile basic diet. By forcing a drop of the liquid at a time from the needle and holding it near the mouth parts of the roaches, the insects soon learned to take the solution rapidly without any loss through spilling. Dr. Penner reports that he has kept flies alive for several months on nothing more than a molar

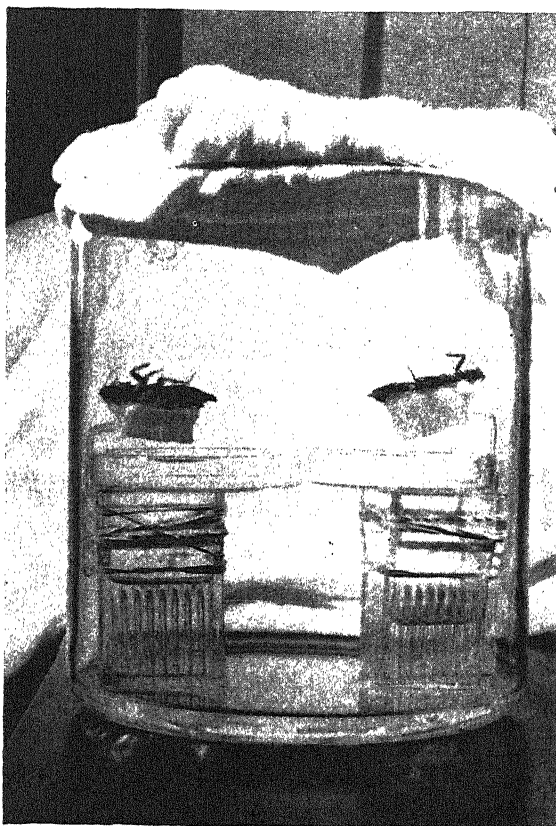


FIG. 2. THE METHOD OF STORING THE MOUNTED INSECTS FOR EXPERIMENTAL WORK

sucrose solution fed in this manner. The fluid intake varied considerably between insects, but in general each roach would consume an average of between 0.2 and 0.3 ml per day, all in a single morning feeding. The quantity of fluid ingested tended to decrease the longer the roach remained mounted. Trying to overfeed them always resulted in prompt regurgitation, an undesirable reaction which we soon learned to control.

There is some evidence that storage of these roaches at about 30 C may keep them more active with a resulting increase in food intake. This is desirable if frequent stools are to be passed. Experiments are also in progress in which

we are attempting to feed more solids in the hope that we may be able to increase the number and the quantity of stools passed.

Stools were transferred from the moist agar surface and were emulsified in several drops of nutrient broth. Streak plates were then made from the emulsion on differential media, and isolations were fished for pure culture identification. Normal flora studies revealed that the species of aerobic organisms in the normal stools of this particular species of roach are few in number, a factor which simplified our later studies with pure cultures of organisms fed to the subjects. Further confirmation of normal stool flora for these insects is in progress together with controlled feedings of cultures. The results of these investigations will be reported in a future paper.

#### ACKNOWLEDGMENT

The authors are indebted to Carl D. Brandt, a junior at the University of Connecticut, who prepared the photographs for this paper.

#### SUMMARY

A relatively simple technique for controlled feeding of insects being employed in microbiological studies is presented. Experiments designed to study both the normal and the induced flora of stools from such insects can be materially advanced by the elimination of ordinary body contaminants. A subsequent paper will outline the results of studies now in progress on attempts to pass specific organisms through the alimentary tract of *Blaberus craniifer*, a large roach common in Florida. Similar techniques may be applied with flies and other insects to see whether they are capable of serving as carriers of specific organisms over a period of time.

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## AN ACTINOPHAGE FOR STREPTOMYCES GRISEUS<sup>1,2</sup>

H. CHRISTINE REILLY, DALE A. HARRIS, AND SELMAN A. WAKSMAN

*New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey*

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Insufficient differentiation is frequently made between the production by a microorganism of an autolytic principle, or an agent which dissolves the cell of the organism producing it, and a phage or transferable principle, which is capable of dissolving not only the cells of the culture producing it but also those of other cultures of the same or other organisms upon transfer. This has often led to confusion in the interpretation of certain stages in the life cycle of the organism or of some of its metabolic processes. Although much light has been thrown in recent years on the nature and mode of action of phages of bacteria, the production of such agents by other microorganisms, notably fungi and actinomycetes, is still insufficiently understood. The significance of the ability of a phage or a viruslike agent to attack filamentous microorganisms in the practical utilization of such organisms for the production of various chemical agents has recently been emphasized in the discovery of a phage which has the capacity to attack streptomycin-producing strains of *Streptomyces griseus*.

The term "bacteriophage" is usually applied to the virus or phage of bacteria, and the term "mycophage" to that of fungi. By analogy, the term "actinophage" may be used to designate the phage of actinomycetes. The origin of the phage, whether it is carried in the culture or brought in from outside like any other contamination, its nature and activity, and its similarity to bacterial phages form some of the most important problems in the elucidation of this natural phenomenon.

### HISTORICAL

The first recorded observations of the lysis of an actinomycetes culture and of the significance of this reaction in the life cycle of the organism and in the production of new strains were made by Dmitrieff (1934) and by Dmitrieff and Soutéeff (1936). A culture of an organism called by the authors *Actinomyces bovis*, and evidently belonging, according to modern concepts, to the genus *Streptomyces*, was found to undergo lysis in various media. When the culture was grown on agar media, the production of lysis was found to be associated only with the formation of a certain type of colony. The organism produced as a result of lysis two types of daughter colonies: one was similar to the mother colony and possessed continued capacity for lysis; the other did not lyse and was morphologically different from the first type. The lysing colonies possessed

<sup>1</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

<sup>2</sup> With partial support from a grant by the Commonwealth Fund of New York.

strong proteolytic properties and apparently did not form any aerial mycelium; the nonlysing colonies were less proteolytic and formed a chalky aerial mycelium, which changed the reaction of litmus milk to alkaline. In broth cultures, lysis took place in 2 to 3 weeks; it was associated with the living organism and was of the nature of a nonenzymatic but nontransmissible lytic factor.

Wieringa and Wiebols (1936) and Wiebols and Wieringa (1936) reported that various actinomycetes isolated from infected potatoes underwent lysis in culture. This phenomenon was believed to be due to the production of specific transmissible phages. Various organisms yielded phages which were active also upon other organisms; thus *A. bovis* produced a phage which was active upon *A. scabies* and *A. farcinicus*. These investigators were thus the first to emphasize the formation by actinomycetes of filterable and transmissible agents comparable to bacteriophages and polyvalent in nature. Their probable role in the control of potato scab in the soil has been suggested.

In view of the taxonomic relationship between the actinomycetes and mycobacteria, it may also be of interest to recall that Steenken (1935) observed lysis among the latter. This did not, however, appear to be a result of phage action. A virulent culture of *Mycobacterium* yielded a nonvirulent strain (R variant) which began to lyse after 3 or 4 months.

Krassilnikov (1938) made a detailed study of the course of autolysis of different actinomycetes isolated from the soil. A well-developed colony on an agar plate gradually became slimy, flat, and transparent. When transferred to a fresh medium, the colony either failed to develop or produced a much-delayed growth. Autolysis did not occur over the whole surface of the colony, but took place in sectors or spots; frequently it began in the center and spread toward the periphery. This phenomenon appeared to be very general among parasitic organisms and occurred less commonly among the saprophytes.

Krassilnikov and Koreniako (1939) emphasized the resemblance of the process of autolysis among actinomycetes to the Twort phenomenon, or phage production by bacteria. They reported that the lytic factor of actinomycetes, contrary to the observations of Wieringa and Wiebols, was highly specific, since it had no action on other species or even on other strains of the same species of *Actinomyces*. Lysis took place when growth of the organism was delayed for one reason or another or at the time of aging of the culture. Since different cultures underwent lysis with varying degrees of rapidity, it was assumed that the quantitative production of the lytic factor or its mode of action was distinct with different organisms. At high temperatures (60 to 70 C), lysis occurred in a few minutes. The lytic agent was resistant to 80 C for 1 hour, but was destroyed at 100 C in 5 minutes. Not only living but also dead cells were affected, thus showing a difference in action from that of true phage.

Katznelson (1940) isolated from manure composts a thermophilic culture of an actinomyces which underwent rapid lysis at 50 C when grown on starch ammonium sulfate agar media; no transmissible lytic agent could be demonstrated. Schatz and Waksman (1945), studying the production of streptomycin by different strains of *S. griseus* obtained from colonies of a given culture, observed

that colonies devoid of aerial mycelium produced no streptomycin. Such colonies gave rise to cultures which underwent much more rapid lysis than the normal cultures producing aerial mycelium. In the practical production of streptomycin it is generally observed that under submerged conditions of growth maximum formation or accumulation of the antibiotic corresponds to the beginning of lysis; advanced lysis usually results in a rapid destruction or inactivation of the streptomycin already produced.

Although these meager series of observations seemed to point definitely to the capacity of some actinomycetes to produce phagelike agents under certain conditions of culture, they threw very little light upon the nature and activities of these agents. They were not even sufficiently differentiated from lytic reactions due to enzymelike mechanisms.

The problem of phage production by actinomycetes entered a new phase with the discovery that the streptomycin-producing strains of *Streptomyces griseus* are subject to attack by a virus or a phagelike agent. This reaction appeared to be quite distinct from the lytic phenomenon mentioned above.

Saudek and Colingsworth (1947) were the first to report the production by *S. griseus* of a transmissible lytic agent which had all the properties of phage. The phage developed in the presence of young cultures of *S. griseus*. These workers used the plaque method with a phage-sensitive strain of *S. griseus* for measuring the concentration of the phage. Streptomycin production was partly or completely prevented by the phage. Cultures resistant to the phage could easily be isolated.

This problem was independently investigated by Woodruff (1947). When a submerged culture of *S. griseus* was placed in a stationary condition, with plugs removed from the flask, and exposed to laboratory air for 24 hours, the freshly formed pellicle showed evidence of plaque formation. The same phenomenon was observed in a factory 500 miles away. Multiplication of the phage took place upon each transfer of a filtered culture into a fresh culture of *S. griseus*. After six transfers, each phage particle increased to  $75 \times 10^{20}$  particles. The phage was active against all streptomycin-producing strains of *S. griseus* but not upon the non-streptomycin-producing strains. The culture produced phage-resistant strains readily. These retained the capacity of producing streptomycin but were not free from phage. The actinophage had properties similar to bacterial phages, such as those of *Escherichia coli*, as shown both by cultural characteristics and by appearance in photographs made by means of an electron microscope.

It has thus been established beyond doubt that at least certain species of *Streptomyces* can be attacked by a true phage. In view of the possible importance of this phenomenon in streptomycin production, and also in order to throw light upon its significance in the life cycle of the organisms producing the phage and in the taxonomy of actinomycetes as a whole, a detailed study was undertaken of the production, nature, and activity of this phage. Certain of the more immediate problems were at first investigated. These included the sensitivity of various strains of *S. griseus* to actinophage, the effect of actinophage upon the

growth and streptomycin production by *S. griseus* in static and in submerged culture, multiplication of active phage under different conditions of culture of *S. griseus*, effect of temperature upon phage activity, and the action of *S. griseus* phage upon non-streptomycin-producing strains of this organism and upon other actinomycetes.

#### EXPERIMENTAL METHODS AND RESULTS

*Cultures used.* A number of strains of *S. griseus* were used. These included several original isolations of streptomycin-producing cultures and a number of active and inactive strains obtained from them by colony selection. In addition, other strains of *S. griseus* not producing any streptomycin and other actinomycetes taken from the culture collection or freshly isolated from various substrates were also investigated.

The more important cultures are listed here.

(1) Streptomycin-producing strains of *S. griseus*:

*S. griseus* 3463, the original streptomycin-producing culture 1S-16.

*S. griseus* 3480, an original culture isolated independently.

*S. griseus* 3481, another original isolate.

*S. griseus* nos. 4 and 9, strains isolated from culture 3463.

*S. griseus* 3475, a strain isolated from culture no. 4.

*S. griseus* 3523 and 3524, streptomycin-producing cultures comparable to nos. 4 and 9.

*S. griseus* 3475-2PR, a phage-resistant culture obtained from 3475.

(2) Non-streptomycin-producing cultures of *S. griseus*:

*S. griseus* 3478, a culture producing grisein.

*S. griseus* 3326, the original culture of *A. griseus* isolated in this laboratory in 1915 and kept on artificial media since then.

*S. griseus* 3326a, the same culture as above, which was deposited with the Central-bureau in Holland in 1920 and recently received from that collection.

*S. griseus* 3522, culture isolated by Bucherer and also received from Holland.

*S. griseus* 3495, culture isolated from no. 4; it does not produce streptomycin, but forms another still unidentified antibiotic.

(3) Other cultures:

*Streptomyces bikiniensis*, a streptomycin-producing organism distinct morphologically and culturally from *S. griseus* and isolated from a Bikini soil (Johnstone and Waksman, 1947).

*Streptomyces violaceus-ruber*, a culture isolated from the soil and kept for many years in the collection.

*Phage used.* A phage preparation, originally obtained from Merek and Company and designated as M, was used in all these investigations.

*Assay methods.* In preliminary experiments, 0.1-ml portions of *S. griseus* culture filtrate containing the phage were added to 10-ml portions of nutrient agar; these were poured into petri plates and allowed to solidify. Aqueous spore suspensions of different strains of *S. griseus* were streaked on the surface of the plates and incubated at 28 C for 48 hours. The growth of strains nos. 4 and 9 was completely inhibited. Strain 3475 showed a few small colonies on the plate. As these appeared to be resistant to the action of the phage, they were picked from the plates and inoculated upon fresh agar slants. Several cultures were

obtained from the resistant colonies. One was selected for further study and designated as 3475-2PR.

To assay the phage preparations quantitatively, 10-ml portions of nutrient agar were poured into a series of five petri plates. These were streaked with spore suspensions of different strains of *S. griseus* and incubated for 24 hours. The diluted phage preparations were then poured over the plates. Upon further incubation at 28 C, the surface growth of the actinomyces streaks showed numerous plaques (figure 1). These were difficult to count. The results obtained

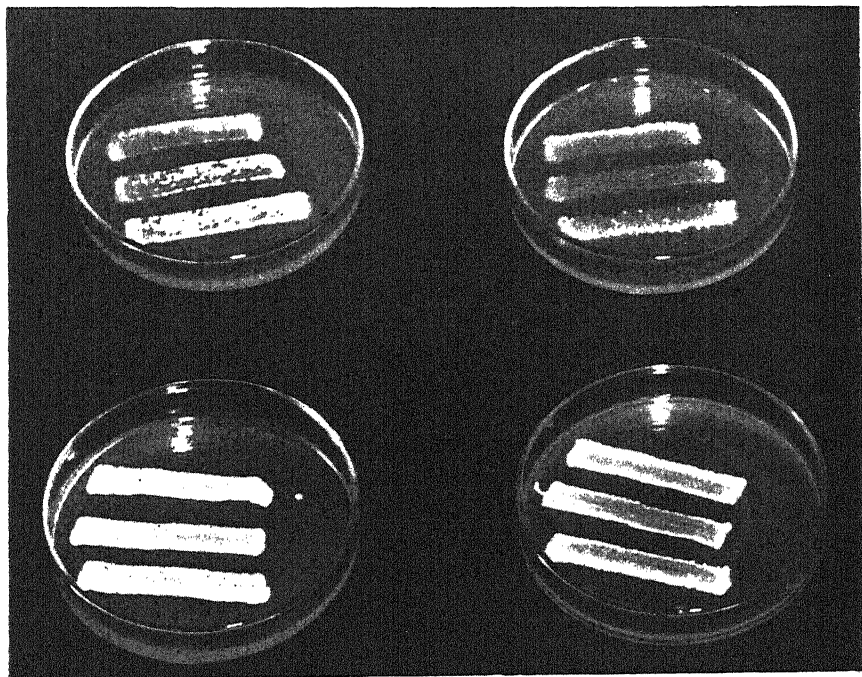


FIG. 1. EFFECT OF ACTINOPHAGE UPON THE DEVELOPMENT OF DIFFERENT STRAINS OF *S. GRISEUS*

Top: Phage-treated; bottom: controls. Left pair: 3475-2PR; right pair: 3475.

were only approximate and need not be reported here, since they were qualitative rather than quantitative.

When the phage was added simultaneously with the inoculum to the fresh medium, growth of the organism was completely prevented; but when the phage was added to cultures which had already been well sporulated, no phage multiplication occurred. These results prove emphatically that the actinophage acts best upon young cultures, as already emphasized by others (Woodruff, 1947). Various streptomycin-producing strains of *S. griseus* appeared to respond differently to the action of the phage, some being less affected than others. When colonies were picked from the agar streak that had been infected with

phage, they produced cultures that were especially resistant to the action of the phage, although they were still capable of supporting considerable phage growth.

The following method was finally adapted for assaying the concentration of phage in a given preparation. A 5-day-old shaken culture of a streptomycin-producing strain of *S. griseus* (no. 3463 being used mostly for this purpose) was filtered aseptically through paper and used as the source of culture material for inoculation of plates. The phage preparation, designated as M-1, was obtained by inoculating the M phage into young cultures of *S. griseus*, which were allowed to incubate for 24 to 72 hours and then were passed through a Seitz filter. A

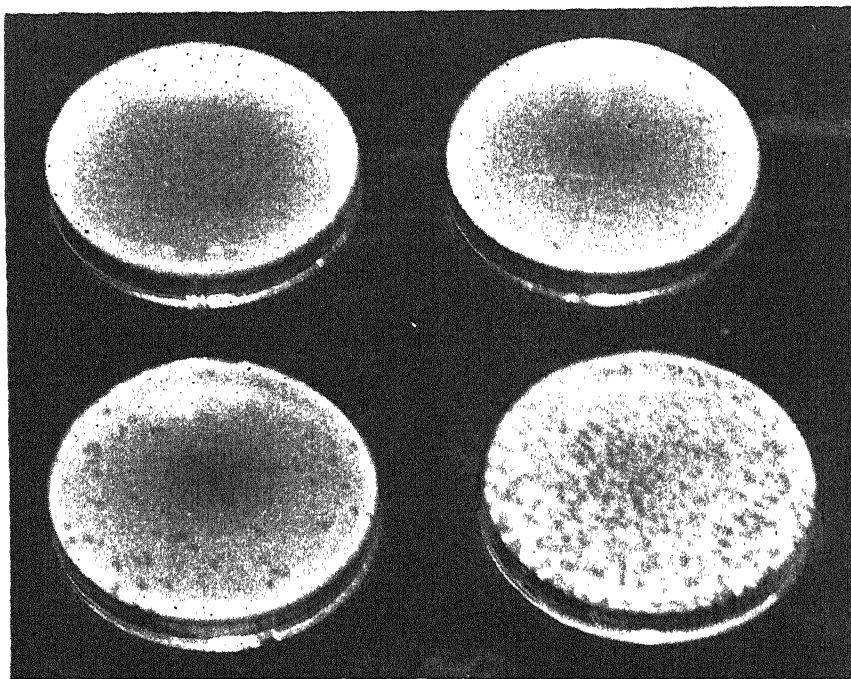


FIG. 2. PLAQUE FORMATION BY ACTINOPHAGE ON PLATES OF *S. GRISEUS*

Top: left to right, control and phage diluted 1:100,000; bottom: left to right, phage diluted 1:10,000 and 1:1,000.

series of dilutions of phage, ranging from  $1:10^6$  to  $1:10^{12}$ , were added to 10-ml portions of sterile nutrient agar, which had been inoculated with 0.1-ml portions of the paper-filtered *S. griseus* culture. The agar portions were poured into plates, mixed thoroughly, and incubated at 28 C for 2 days. The plaque counts were then made, as shown in figure 2. The M-1 phage preparation contained  $4.2 \times 10^{10}$  particles per ml.

This method of assay gave accurate and reproducible results. The first phage preparation, M-1, was kept in the refrigerator and used as a standard. This procedure was now standardized, especially in regard to the effect of size of inoculum and of temperature of incubation. Three different amounts of filtered



7-day-old shaken culture of no. 3463 were added to nutrient agar to give final 10, 1, and 0.1 per cent concentrations. They were inoculated with different dilutions of M-1 phage and incubated at 28 C for 48 hours. The following results were obtained:

Culture inoculum per 100 ml of agar	Plaque counts $\times 10^7$
10.0	391
1.0	698
0.1	756

These results show that a lower inoculum gave higher counts; a 1 per cent inoculum was, therefore, adopted for all subsequent work.

In studying the effect of temperature of incubation upon plaque development, two temperatures were used, 28 C and 37 C. Normal plaque development took place at the lower temperature. No plaques appeared on the plates incubated at 37 C. When these plates were subsequently placed at room temperature for an additional 24 hours, plaques were rapidly produced with 1 per cent inoculum. None of the plates inoculated with 10 per cent of culture material produced any plaques, which points to the fact that not only is a temperature of 37 C unfavorable for phage multiplication, but at that temperature an excess inoculum exerts a destructive, or at least an adsorptive, effect upon the phage.

*Effect of the phage on growth and streptomycin production by S. griseus.* In a preliminary experiment on the effect of phage upon stationary cultures grown in standard medium for streptomycin production,<sup>3</sup> it was found that when the phage was placed in drops upon 2- and 3-day-old pellicles and allowed to incubate further at 28 C, many clear patches were produced in the pellicles, especially in the younger ones. Further investigation indicated that the study of the effect of phage upon growth of *S. griseus* and upon streptomycin production could best be conducted in submerged cultures. This is brought out in table 1. When the phage was added at the time of inoculation of the cultures, very little streptomycin was produced at the earlier periods of incubation, namely, after 3 and 4 days. When the cultures were allowed to incubate further, active streptomycin production occurred, as shown by the 5- and 6-day readings. This is a result, no doubt, of the development of resistant strains in the culture upon continued incubation.

A comparative study of the effect of phage on streptomycin production by strain 3475 of *S. griseus* and by the phage-resistant culture 3475-2PR, isolated from the foregoing strain, under submerged and stationary conditions of growth, tends to confirm the observation above. This is brought out in table 2. After 3 and 4 days' incubation in shaken cultures, no streptomycin was produced by the original strain in the presence of phage; however, after 8 days streptomycin production occurred, the activity of the low phage inoculum equaling that of the controls. The resistant strain, on the other hand, gave good streptomycin

<sup>3</sup> This medium contained 1 per cent glucose, 0.5 per cent each of peptone, meat extract, and NaCl in tap water.

activity during the early incubation period, the presence of phage exerting only a slight depressive effect upon the total activity. Similar results were obtained in stationary cultures, the recovery of the streptomycin-producing capacity in the phage-containing cultures being much slower, however. Under these conditions, the phage-resistant strain did not produce so much streptomycin as the original culture.

TABLE 1  
*Influence of phage on streptomycin production in submerged culture*

PHAGE ADDED*	INCUBATION, DAYS			
	3	4†	5	6†
	Streptomycin produced, µg/ml			
0	21	100	99	133
+	5	8	78	56

\* One ml of phage, containing  $4 \times 10^{10}$  particles per ml, was added to 60-ml portions of glucose peptone meat extract NaCl medium in 250-ml Erlenmeyer flasks inoculated with spore suspensions of *S. griseus*.

† Cultures were kept static for 24 hours, then placed in a shaking machine.

TABLE 2  
*Effect of addition of phage upon growth and streptomycin production by original *S. griseus* and by a phage-resistant strain†*

PHAGE ADDED		STREPTOMYCIN, $\mu\text{g/ML}$ , PRODUCED AFTER DAYS					
After incubation	Phage per ml, $\times 10^7$	Submerged cultures			Stationary cultures		
		3	4	8	8	10	24
Strain 3475							
hours							
0	0	32	111	192	140	188	189
Start	0.7	<5	<5	208	<5	<5	113
Start	70	<5	<5	128	<5	<5	93
24	70	<5	<5	122	<5	<5	24
Resistant strain 3475-2PR							
0	0	124	132	172	16	23	79
Start	70	117	108	140	19	50	36

More detailed results of further experiments on phage multiplication and the effect of phage upon streptomycin production under submerged and stationary conditions of culture are reported in tables 3 and 4. When the phage was added to the culture simultaneously with the inoculum, it multiplied rapidly and at first completely prevented streptomycin production; on further incubation, streptomycin production set in rapidly, and later tended to approach that of the control. The only possible interpretation is that the development of a phage-

resistant strain of *S. griseus* occurred in the culture. When the phage was added to the submerged cultures 16 hours after inoculation, its rate of multiplication was much more rapid, because of the greater amount of available mycelium, with a corresponding reduction in streptomycin production. Here again, streptomycin production set in rapidly later, as a result of the development of resistant strains. Similar results were obtained when the phage was added to the

TABLE 3

*Effect of phage upon the production of streptomycin by S. griseus in submerged culture*

PHAGE* ADDED AFTER	INCUBATION, DAYS					
	3		5		7	
	Phage $\times 10^8$	Sm† $\mu\text{g/ml}$	Phage $\times 10^8$	Sm $\mu\text{g/ml}$	Phage $\times 10^8$	Sm $\mu\text{g/ml}$
Control.....	0	94	0	168	0	258
0 hours.....	1	<5	100	<5	208	192
16 hours.....	37	<5	75	5	92	172
2 days.....	177	22	222	18	382	36
4 days.....	—	—	24	122	45	272
6 days.....	—	—	—	—	53	300

\* One-tenth ml of phage preparation, containing  $40 \times 10^8$  particles, was added to each flask containing 60 ml of medium; this is equivalent to  $0.67 \times 10^8$  phage particles per 1 ml of medium. All results are reported per 1 ml of culture.

† Sm = streptomycin.

TABLE 4

*Effect of phage upon the production of streptomycin by S. griseus in stationary culture*

PHAGE* ADDED AFTER	INCUBATION, DAYS					
	9		13		17	
	Phage $\times 10^8$	Sm $\mu\text{g/ml}$	Phage $\times 10^8$	Sm $\mu\text{g/ml}$	Phage $\times 10^8$	Sm $\mu\text{g/ml}$
Control.....	0	306	0	252	0	273
0 hours.....	19	<5	31	16	24	178
16 hours.....	27	<5	38	6	33	79
2 days.....	47	26	588	36	605	94
6 days.....	29	300	38	219	73	300
12 days.....	—	—	0.9	185	0.4	300

\* Same as table 3.

48-hour-old cultures, the effect being magnified, as shown by the more rapid rate of phage development. The small amount of streptomycin formed at the time the phage was added did not increase until the seventh day, when the ability to form streptomycin was apparently recovered. When the phage was added to the 4- and 6-day-old cultures, at a time when growth had reached a maximum, there was a very limited amount of phage multiplication, and little effect was exerted on the streptomycin that had already been produced in the medium.

The results obtained under stationary conditions fully confirmed the results on the submerged cultures, namely, that phage multiplication was at a maximum when added to the 2-day-old cultures, that the addition of phage at the time of inoculation or soon afterward represses streptomycin production, that this is

TABLE 5

*Effect of phage upon the growth, phage multiplication, and streptomycin production by different actinomycetes in stationary cultures*

ORGANISM	PHAGE ADDED*	9 DAYS		13 DAYS	
		Phage per ml $\times 10^7$	Sm $\mu\text{g/ml}$	Phage per ml $\times 10^7$	Sm $\mu\text{g/ml}$
Streptomycin-producing strains of <i>S. griseus</i> No. 3463	0	—	—	0	21
	+	—	—	200	5
No. 3475	0	0	30	0	180
	+	>50	<5	370	<5
No. 3480	0	0	31	0	189
	+	10	<5	30	28
No. 3481	0	0	73	0	174
	+	50	<5	260	13
No. 4	0	0	43	0	201
	+	30	<5	160	<5
3475-2PR	0	>0.01	40	40	129
	+	>50	16	370	75
<i>S. griseus</i> 3478	0	0	<5	0	<5
	+	0	<5	0	<5
<i>S. griseus</i> 3326a	0	—	—	0	<5
	+	—	—	<0.2	<5
<i>S. bikiniensis</i>	0	0	<5	0	30
	+	3	30	7	33

\* Each 60-ml flask of culture received at start 0.1 ml of M-1 phage, amounting to  $7 \times 10^7$  particles per 1 ml of medium.

followed by the development of resistant strains which result in a considerably delayed formation of the antibiotic, and that, when added to older cultures, some phage development occurs with little effect upon the streptomycin present in the culture.

*Effect of phage M-1 upon different strains of S. griseus and upon other actinomycetes.* A detailed study of the effect of phage upon different cultures of actinomycetes, comprising different species and strains, brought out the fact (tables 5 and

6) that phage M-1 affected all the streptomycin-producing strains of *S. griseus*; it inhibited streptomycin production, and it multiplied at the expense of the growth of the organism. It had little effect upon the growth of other organisms. The two non-streptomycin-producing strains of *S. griseus* as well as some of the other actinomycetes tended to destroy or adsorb the phage, the mechanism of

TABLE 6

Phage multiplication in shaken cultures of various actinomycetes and its effect upon the production of antibiotics

ORGANISM	PHAGE* ADDED AFTER HOURS OF INCUBATION	TOTAL INCUBATION, DAYS				
		2	4	Antibiotic activity	6	Antibiotic activity
		Phage per ml $\times 10^7$	Phage per ml $\times 10^7$	<i>S units/ml</i>	Phage per ml $\times 10^7$	<i>S units/ml</i>
<i>S. griseus</i> no. 4.....	Control†	0	0‡	66	0	90
<i>S. griseus</i> no. 4.....	Start	22	650	35	930	48
<i>S. griseus</i> no. 4.....	24	9,500	7,000	96	4,600	135
<i>S. griseus</i> no. 4.....	48	—	166	120	90	90
<i>S. griseus</i> 3478.....	Control	0	0	—	—	14
<i>S. griseus</i> 3478.....	Start	8	1.3	—	—	15
<i>S. bikiniensis</i> .....	Control	0	0	29	—	18
<i>S. bikiniensis</i> .....	Start	0.05	0.13	24	0	30
<i>S. lavendulae</i> .....	Control	—	0	15	—	<10
<i>S. lavendulae</i> .....	Start	—	8.8	<10	—	<10
<i>S. violaceus-ruber</i> ....	Control	—	—	—	0	—
<i>S. violaceus-ruber</i> ....	Start	—	—	—	0.00002	—
<i>Nocardia asteroides</i> ..	Control	—	—	—	0	—
<i>Nocardia asteroides</i> ..	Start	—	—	—	9.4	—
<i>Micromonospora</i> sp...	Control	—	—	—	0	—
<i>Micromonospora</i> sp...	Start	—	—	—	9.1	—

\*  $70 \times 10^6$  phage particles added per ml of culture.

† No phage was added to control cultures.

‡ One plaque appeared on one plate; there is some doubt as to whether this plaque was due to phage or was due to the growth of an *S. griseus* colony that was antagonistic to the test organism.

this reaction still being uncertain. The phage had no injurious effect either upon growth or upon streptomycin production of *S. bikiniensis*.

The foregoing results were confirmed by a number of other experiments, with minor variations. For example, no. 3495, a strain of *S. griseus* isolated from a streptomycin-producing culture, which does not form streptomycin but does form another antibiotic inactive against *Escherichia coli* but active against gram-positive bacteria, gave no phage multiplication but showed occasionally a

change in the nature of the antibiotic spectrum. *S. bikiniensis* allowed no phage multiplication, or actually brought about the disappearance of the phage, and showed at times increased streptomycin production in the presence of the phage. The latter reaction may have been due to nutritive effects of certain constituents of the phage preparation.

In a series of experiments upon phage multiplication in cultures of different strains of streptomycin-producing *S. griseus*, each of 10 such strains was inoculated into four 60-ml portions of broth in 250-ml Erlenmeyer flasks. To two flasks of each series, 0.1-ml portions of phage M-1 were added at the time of inoculation; two flasks were left as controls. Phage determinations were made after 2, 4, and 6 days of incubation at 28 C. The antibiotic potency of the cultures was determined by the usual cup technique against a streptomycin standard (table 7).

There was considerable variation among the different cultures both in the extent of phage multiplication and in the rapidity of recovery of streptomycin-producing potency. As in previous experiments, the phage-resistant culture 3475-2PR showed comparatively little effect of the phage upon streptomycin production.

*Effect of temperature upon phage.* In a preliminary experiment on the effect of temperature upon actinophage, several 5-ml portions of phage M-1, diluted to give  $43 \times 10^7$  particles per ml, were placed in sterile test tubes and kept in a water bath at four different temperatures for 10 minutes. No significant destruction of the phage took place at 40 to 65 C; a definite reduction occurred at 75 to 80 C; and maximum destruction was reached at 100 C.

In a more carefully controlled experiment, similar dilutions of phage were made. They were placed in water baths and incubated at various temperatures for 10 minutes and for 1 hour (table 8). The phage was stable for 1 hour at 65 to 75 C. Appreciable reduction in the number of phage particles occurred in the tubes kept for 10 minutes at 85 to 90 C and a further decrease occurred upon continued incubation. Heating for 1 hour at 90 C was not sufficient, however, to destroy the phage completely.

To determine the effect of temperature with prolonged storage upon the survival of phage, several 10-ml portions of phage M-1 diluted 1:100 with sterile water were added to test tubes, stoppered with sterile rubber stoppers, and placed at four different temperatures. After several periods of incubation, phage determinations were made. The results show (table 9) that incubation for 3 days at 56.5 C brought about an appreciable decrease in phage concentration; after 12 days at this temperature more than 99 per cent of the phage was destroyed. At 37 C the decrease was much slower, incubation for 12 days giving about 58 per cent loss of phage concentration and nearly complete loss after 29 days. At 28 C there was a small decrease after 12 days' incubation and marked decrease after 29 days. There was no change in concentration of the phage at 6 C on continued incubation.

Further studies on the effect of temperature upon phage multiplication confirmed the previous results. The optimum was at 28 C. There was no increase

in phage content at 37 C, and at 56.5 C more than 97 per cent of the phage was destroyed in 1 day. The extent of phage multiplication depended largely upon the size of the inoculum. The greater the number of cells of *S. griseus* present in the culture, the greater was the amount of phage produced. At 56.5 C, the size

TABLE 7

*Multiplication of phage in shaken cultures of streptomycin-producing strains of S. griseus*

STRAIN NO.	ADDITION OF PHAGE	DAYS OF INCUBATION			
		2		4	6
		Phage* per ml $\times 10^7$	Sm	Sm	Sm
			$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
No. 3463 .....	0	0	17	90	116
No. 3463 .....	+	84	<5	<5	34
No. 3464 .....	0	0	<5	28	20
No. 3464 .....	+	70	<5	<5	18
No. 4 .....	0	0	<5	15	12
No. 4 .....	+	15	<5	<5	<5
No. 9 .....	0	0	<5	>50	51
No. 9 .....	+	64	<5	<5	51
No. 3475 .....	0	0	<5	64	92
No. 3475 .....	+	83	<5	<5	76
No. 3498 .....	0	0	23	49	78
No. 3498 .....	+	76	<5	<5	16
No. 3499 .....	0	0	<5	43	90
No. 3499 .....	+	20	<5	<5	<5
No. 3523 .....	0	0	21	114	149
No. 3523 .....	+	81	<5	<5	20
No. 3524 .....	0	0	17	57	72
No. 3524 .....	+	146	<5	7	32
No. 3475-2PR .....	0	<0.001	<5	98	104
No. 3475-2PR .....	+	8	<5	42	104

\* The cultures treated with phage contained at start  $4 \times 10^7$  phage particles per ml.

of inoculum also had an effect upon the extent of phage destruction: the larger the inoculum, the lower was the rate of phage destruction. The nature of the medium in which the phage was suspended had a marked influence upon the rate of its destruction at 56.5 C. The phage suspended in water showed only about 40 per cent destruction in 1 day, whereas the phage placed in broth lost more than 99 per cent of its activity in the same time. After 3 days, the phage

diluted with broth was completely destroyed, whereas considerable phage was left in the aqueous suspension, although marked destruction had taken place.

*Multiplication of actinophage in the presence of living and dead cells of S. griseus.* Finally, studies were made upon the ability of the phage to multiply at the ex-

TABLE 8  
*Effect of temperature upon the stability of actinophage*  
(At start,  $43 \times 10^7$  phage particles per ml)

TEMPERATURE	PHAGE $\times 10^7$ PER ML, AFTER	
	10 minutes	1 hour
C		
Control	43	
65	45	45
75	—	45
85	0.07	0.0003
90	0.001	0*

\* The actual count was 5 particles per ml.

TABLE 9  
*Stability of phage in aqueous suspension upon storage at several temperatures*

TEMPERATURE OF STORAGE	PHAGE PARTICLES $\times 10^7$ PER ML, AFTER STORAGE*		
	3 days	12 days	29 days
C			
6	44	—	60
28	31	20	0.00005
37	37	15	0.0000009
56.5	18	0.001	0

\* At start all preparations contained  $36 \times 10^7$  particles of phage per ml.

TABLE 10  
*Multiplication of actinophage in living and dead cultures of S. griseus*

S. GRISEUS CULTURE	PHAGE $\times 10^7$		
	Start	1 day	2 days
Living.....	43	136	189
Dead.....	65	76	58

pense of living and dead cultures of *S. griseus*. Two 10-ml portions of a 40-hour-old shaken culture of a streptomycin-producing strain (no. 3475) were transferred aseptically to sterile test tubes. One tube was placed in a water bath at 75 C for 10 minutes, to kill the spores and mycelium of *S. griseus*; the second tube was not heated. To both tubes were added 0.1-ml portions of phage M-1; the tubes were incubated at 28 C and phage concentrations determined at the start and



after 1 and 2 days. The results presented in table 10 show that no multiplication of the phage took place in the presence of dead cells of *S. griseus*; in the presence of living cells, a fourfold increase in phage concentration occurred.

#### SUMMARY

The results obtained by Saudek and Colingsworth and by Woodruff on the production of phage by streptomycin-producing cultures of *Streptomyces griseus* have been fully confirmed.

Certain cultures of *S. griseus* are subject to attack by a virus which can be designated as "actinophage."

This phage attacks only the streptomycin-producing strains of *S. griseus*; it has no effect on other streptomycin-producing organisms.

In cultures of strains of *S. griseus* that do not produce streptomycin, the phage does not multiply and may actually be destroyed or adsorbed.

The actinophage of *S. griseus* multiplies only at the expense of living cell material and not upon the heat-killed material of this organism.

Phage-sensitive cultures of *S. griseus* give rise rapidly to strains which are resistant to the action of the phage.

The actinophage has an optimum temperature for multiplication at 28 C. It does not multiply at 37 C or above.

Actinophage can withstand a temperature of 75 C for 1 hour, but is completely destroyed at 100 C in 10 minutes.

The actinophage can be stored at 6 C without loss of activity, but storage at 28 C or at higher temperatures results in a loss of activity, the rate of loss being proportional to the temperature.

The nature of the medium in which the actinophage is suspended greatly influences the rate of its destruction.

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## TWO STREPTOMYCIN-RESISTANT VARIANTS OF MENINGOCOCCUS<sup>1</sup>

C. PHILLIP MILLER AND MARJORIE BOHNHOFF

*Department of Medicine, University of Chicago, Chicago, Illinois*

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One of the striking differences between penicillin and streptomycin is the rapidity with which microorganisms develop resistance to the latter. Resistance to penicillin can be acquired, but always in relatively small increments at each subcultivation on artificial media or in each passage of the strain through an experimental animal. A high degree of resistance can be attained *in vitro* or *in vivo* only by repeated exposure to increasing concentrations of the drug.

Miller and Bohnhoff (1947a), for instance, found that 147 transfers onto media containing increasing concentrations of penicillin raised the resistance of a strain of meningococcus sufficiently to permit it to grow abundantly on media containing 5,000 units per ml. They have also shown (Miller and Bohnhoff, 1946b, 1947b) that the resistance of a strain of meningococcus could be increased by serial passage through mice treated with subcurative doses of penicillin. They used cultures of hearts' blood as inocula for each succeeding animal passage. The dose required to protect approximately half of the mice rose from 10 units to 1,700 units in the course of 61 passage inoculations.

Resistance to streptomycin, on the other hand, was found to develop with such rapidity that two or three transfers onto media containing increasing concentrations sufficed to permit meningococcus or gonococcus to multiply on media containing 50,000  $\mu$ g of streptomycin per ml (Miller and Bohnhoff, 1946a). Meningococci which were rendered streptomycin-resistant by this means retained approximately the virulence of the original parent culture and were resistant to streptomycin *in vivo*. Mice inoculated with such resistant meningococci died in spite of doses of streptomycin which would have protected them against infection with normal meningococci.

The present communication presents evidence that this rapid development of streptomycin resistance by meningococcus is due to the selective propagation of resistant variants which become apparent during growth on streptomycin-containing media. These variants are presumed to originate from streptomycin-resistant mutants which are arising regularly in the bacterial population of the normal parent strain before its exposure to the drug. In the course of these experiments, a second variant has been encountered which is not only resistant to streptomycin but is actually dependent on streptomycin for its growth

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*in vitro* and *in vivo*. Both variants have developed from each of 18 strains of meningococcus.<sup>2</sup>

#### METHODS

*Strains of meningococcus.* The 18 strains used in these experiments included (a) old stock strains which have been under cultivation in the laboratory for several years, (b) strains recently isolated from cases of epidemic meningitis, and (c) strains isolated from the nasopharynx of healthy carriers. Each of the strains was definitely identified as a member of one of the 3 fixed types: I, II, or II alpha. All of the strains produced colonies typical of meningococcus and fermented only glucose and maltose.

*Media.* The medium used most commonly throughout these experiments has been described in a previous communication as casein digest agar (Miller and Bohnhoff, 1947a). Several other media were employed at various times for purposes of comparison and to make certain that the results obtained were not dependent on any ingredient of the medium itself. Media thus employed were meat digest cysteine agar (Miller and Bohnhoff, 1947a), Difco nutrient agar, brain heart infusion agar, and proteose peptone no. 3 agar.

The media were usually enriched by the addition of fresh, defibrinated sheep or rabbit blood. A few experiments were conducted with agar containing rabbit serum.

When a liquid medium was required trypticase soy broth<sup>3</sup> was used.

*Preparation of streptomycin media.* A total of 25 preparations of streptomycin<sup>4</sup> have been used. They were obtained from seven manufacturers and varied widely in streptomycin activity but included some preparations of an especially high degree of purity.

Plates of streptomycin agar were made up as follows and were always used within a few hours of preparation: a saline solution of streptomycin was diluted to convenient concentrations and 1 ml of appropriate dilutions pipetted into each petri dish. Five-tenths ml of fresh, defibrinated blood were then put beside it. Melted agar (cooled to 45 C) was added, and the contents of each plate were thoroughly mixed.

*Method of inoculation of the streptomycin plates.* When heavy seedings of meningococci were to be planted onto a series of plates containing graded concentrations of streptomycin, the following technique was employed because it distributed the inocula evenly and did not break the surface of the agar:

After the agar had set, 5 small glass balls (about 6 mm in diameter), such as

<sup>2</sup> Our preliminary communication (Miller and Bohnhoff, 1947c) reported that these variants developed from 16 of 18 strains. The 2 strains originally considered failures have been re-examined and found to produce small numbers of both A and B variants.

<sup>3</sup> Baltimore Biological Laboratory.

<sup>4</sup> Preparations of streptomycin were supplied by the Antibiotics Study Section of the National Institute of Health, U. S. Public Health Service; the Division of Penicillin Control and Immunology, Food and Drug Administration; Abbott Laboratories; Commercial Solvents Corporation; Eli Lilly & Company; Merck & Co.; Chas. Pfizer & Company; E. R. Squibb & Son; and Upjohn Company.

are customarily used for defibrinating blood, were placed on the surface of the agar in each plate. It was found to be convenient to have these "beads" distributed in test tubes, 5 to a tube, before sterilization, so that the whole contents of a tube could be rolled out gently onto the agar surface.

The 18-hour growth from an agar culture in an ordinary 16-ounce medicine bottle was harvested in 9.0 ml of gelatin Locke's solution,<sup>5</sup> sedimented by centrifugation, and resuspended in 0.5 ml gelatin Locke's solution. The meningococci were dispersed by drawing the suspension repeatedly into a capillary pipette from which one drop was allowed to fall onto the agar in each plate. These inocula contained approximately  $1.0$  to  $2 \times 10^{10}$  microorganisms. The plates were then stacked in a holder and shaken gently in all directions so that the beads rolled back and forth over the surface of the agar and distributed the inocula uniformly. The beads were then discarded. The plates were incubated for 3 days, the first in a candle jar, and then allowed to stand for a few more days at room temperature. They were all examined carefully each day for 5 or 6 days.

*Mouse inoculations* were made to determine virulence and also streptomycin resistance. A loopful of growth from an 18-hour culture was rubbed up in a few ml of gelatin Locke's solution and the suspension diluted until it reached a density equal to no. 3 of the McFarland series (Kolmer and Boerner, 1945), which experience has shown to contain approximately one billion meningococci per ml. From this standard suspension, 10-fold dilutions were made in 4 per cent mucin<sup>6</sup> and 1 ml quantities injected intraperitoneally into mice weighing 16 to 20 grams (Miller and Castles, 1936).

Mice were treated with streptomycin by the injection of the desired dose in 0.5 ml of saline under the skin of the animal's back.

As many as possible of the mice that died were autopsied, and cultures of hearts' blood were made on casein digest agar and also on the same agar to which 100  $\mu$ g of streptomycin per ml had been added.

#### EXPERIMENTAL RESULTS

The two variants described below appeared when meningococci were inoculated onto media containing streptomycin greatly in excess of that which is considered the optimal bactericidal concentration. Identical results were obtained from cultures started with a single isolated colony and from an ordinary transfer of a stock culture. A heavy seeding of an overnight growth of a normal, sensitive strain of meningococcus was planted onto a series of 8 to 12 plates containing graded concentrations of streptomycin. As most of the experiments were performed with one preparation of streptomycin,<sup>7</sup> the concentrations given below are those of that single preparation. The range varied from 10  $\mu$ g per ml to 10,000  $\mu$ g per ml. The intermediate concentrations were usually 20, 40, 60, 100, 200, 400, 600, 1,000, and 4,000  $\mu$ g per ml.

The growth on a series of 6 plates is shown in figure 1.

<sup>5</sup> Locke's solution containing 0.1 per cent gelatin.

<sup>6</sup> Granular mucin, type 1701-W, supplied by the Wilson Laboratories, Chicago, Illinois.

<sup>7</sup> A preparation marketed for therapeutic use by Eli Lilly & Co.

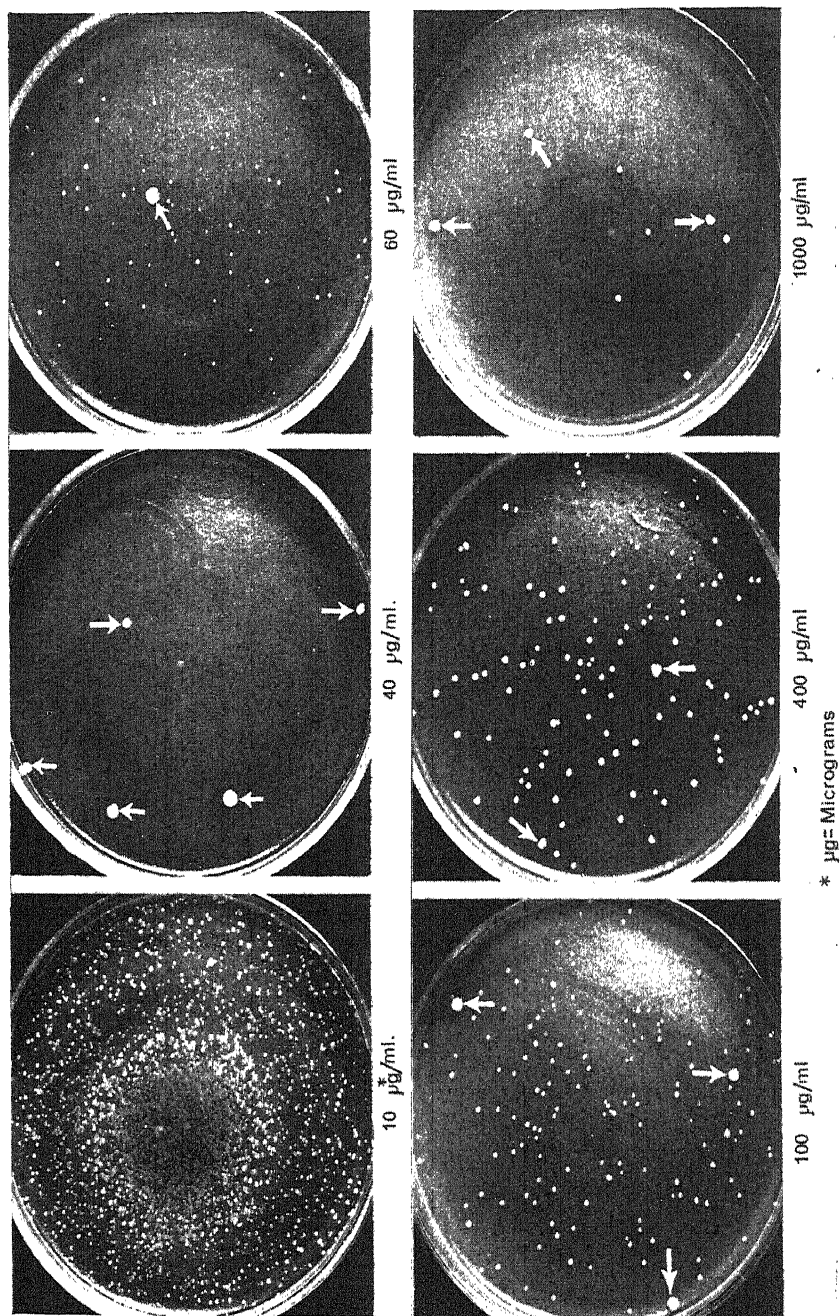


FIG. 1. GROWTH OF MENINGOCOCCUS FROM EQUIVALENT INOCULA ON GRADED CONCENTRATIONS OF STREPTOMYCIN

Photographed after 72 hours' incubation

The first plate (10  $\mu\text{g}$ ) shows only normal meningococcus colonies. The type A colonies are indicated by arrows. The third (60  $\mu\text{g}$ ), fourth (100  $\mu\text{g}$ ), and fifth (400  $\mu\text{g}$ ) plates show numerous type B colonies. The sixth plate (1,000  $\mu\text{g}$ ) shows 5 type B colonies.

After 24 hours' incubation, the plate containing 10  $\mu\text{g}$  per ml showed confluent growth, and the one containing 20  $\mu\text{g}$  per ml a very large number of colonies indistinguishable from normal meningococcus colonies. A few of these normal colonies occasionally appeared on 40  $\mu\text{g}$  per ml, but none on concentrations higher than that.

*Type A variant.* On plates containing 40  $\mu\text{g}$  per ml, a second type of colony was visible at the end of 24 hours' incubation and continued to grow for the next 48 hours, reaching a size of 3 to 5 mm in diameter; i.e., considerably larger than normal meningococcus colonies. It differed from normal colonies in color as well as size, for it acquired a distinctly yellowish tinge which became more marked during the second and third day of incubation and after another day or two at room temperature. This variant, which developed from each of the 18 strains, has been designated type A.

Except on plates containing 10 to 20  $\mu\text{g}$  per ml which were so crowded with normal colonies that they could not be distinguished, type A variants developed in about equal numbers from any given strain on all concentrations of the drug. This number, however, varied from strain to strain. Most strains produced 2 to 5 colonies per plate, an average incidence of approximately 1 to 3 in  $10^{10}$  of original bacterial population. Figure 2 presents the results of 32 experiments performed with one strain (113) and illustrates the uniformity of incidence of type A colonies. One strain, however, developed greater numbers of type A colonies, 5 to 30 per plate.

The type A variants had the following properties: They were highly resistant to streptomycin as they were able to grow on concentrations of the drug as high as 10,000  $\mu\text{g}$  per ml. They were also able to multiply on streptomycin-free media. They retained all of the following properties of the parent strains from which they arose: morphology, staining characteristics, sugar fermentation, virulence for mice, and type specificity as determined by agglutination and by mouse protection tests. Their streptomycin resistance was demonstrated *in vivo* by inoculating mice with mucin suspensions and treating the animals with 15,000  $\mu\text{g}$  in 3 subcutaneous injections of 5,000  $\mu\text{g}$  each at 1, 3, and 5 hours after inoculation. The mice regularly died of meningococcal sepsis, and type A variants were cultured from their hearts' blood.

No loss of streptomycin resistance has been detected in the type A variants either during passage through mice or during subcultivation on streptomycin-free media. Two strains have been transplanted every 5 to 7 days for one year.

Type A variants were found to be slightly more sensitive to penicillin than the parent strain from which they arose.

*Type B variants.* After 48 hours of incubation a second type of variant appeared on all concentrations of streptomycin above 40  $\mu\text{g}$  per ml. After another 24 hours' incubation, additional colonies of this type developed on concentrations of 60 and 100  $\mu\text{g}$  per ml, but no new colonies appeared after 72 hours. The size and color of these colonies varied with the concentration of streptomycin on which they grew. On plates containing 60 to 100  $\mu\text{g}$  per ml, they were very small and light gray; on concentrations above this range, they

were larger and had a distinctly yellowish tinge. On concentrations greater than 400  $\mu\text{g}$  per ml, they resembled the type A colonies in size and pigmentation. The identification of doubtful colonies was made by subcultivation onto streptomycin-free and streptomycin-containing agar.

The number of type B colonies which developed from one type I strain (113) are plotted in figure 3, which shows (a) that the actual numbers varied considerably in different experiments and (b) that they were always most numerous between concentrations of 100 and 400  $\mu\text{g}$  per ml. Curves of the numbers of colonies in individual experiments differed in height but almost always had the shape of the curve of the mean shown in figure 3.

The meningococci composing these type B colonies had the following properties: They were resistant to streptomycin, for they were able to grow on concentrations as high as 5,000  $\mu\text{g}$  per ml. They were dependent on streptomycin for growth; that is, they would grow abundantly from small inocula on concentrations between 100 and 400  $\mu\text{g}$  per ml and would also grow from large inocula on concentrations as low as 5  $\mu\text{g}$  per ml, but they could not be subcultured on media

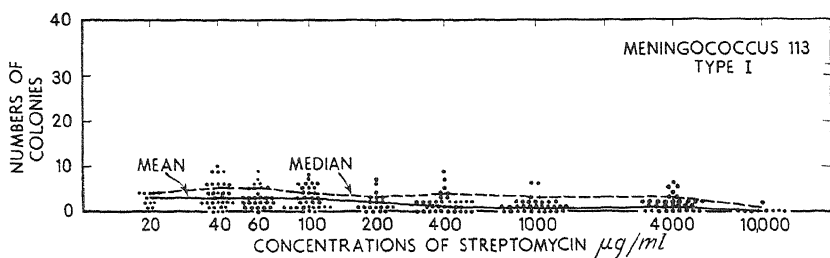


FIG. 2. NUMBERS OF COLONIES OF TYPE A VARIANTS DEVELOPING ON GRADED CONCENTRATIONS OF STREPTOMYCIN

containing less than that minimum of streptomycin. They were nonvirulent for mice unless the mice were treated with streptomycin as described below. They were gram-negative and fermented glucose and maltose when the test media contained 100  $\mu\text{g}$  of streptomycin per ml. They retained the type specificity of the parent strain from which they arose. Rabbit sera prepared against the parent strain conferred protection against experimental infection with these variants in mice treated with streptomycin.

Microscopically, the type B organisms varied somewhat with the concentrations of streptomycin on which they had developed. Preparations made from the small gray colonies grown on 60 or 100  $\mu\text{g}$  per ml showed them to be slightly larger than normal meningococci. Type B organisms growing on higher concentrations in larger pigmented colonies were indistinguishable from normal meningococci. This difference may well be related to the stimulating action of streptomycin mentioned below.

Although the colonial development and microscopic appearance of type B variants differed according to the concentration of streptomycin on which they grew, the identity of all members of this variant was indicated by the following observations: When a type B variant was taken from any concentration and



subcultured onto another concentration, it always grew in colonies of the type regularly produced on that particular concentration. In other words, small gray colonies always developed on concentrations of 60 to 100  $\mu\text{g}$  per ml and large colonies tinged with yellow on concentrations above 200  $\mu\text{g}$  per ml, regardless of the concentration from which the inocula were taken.

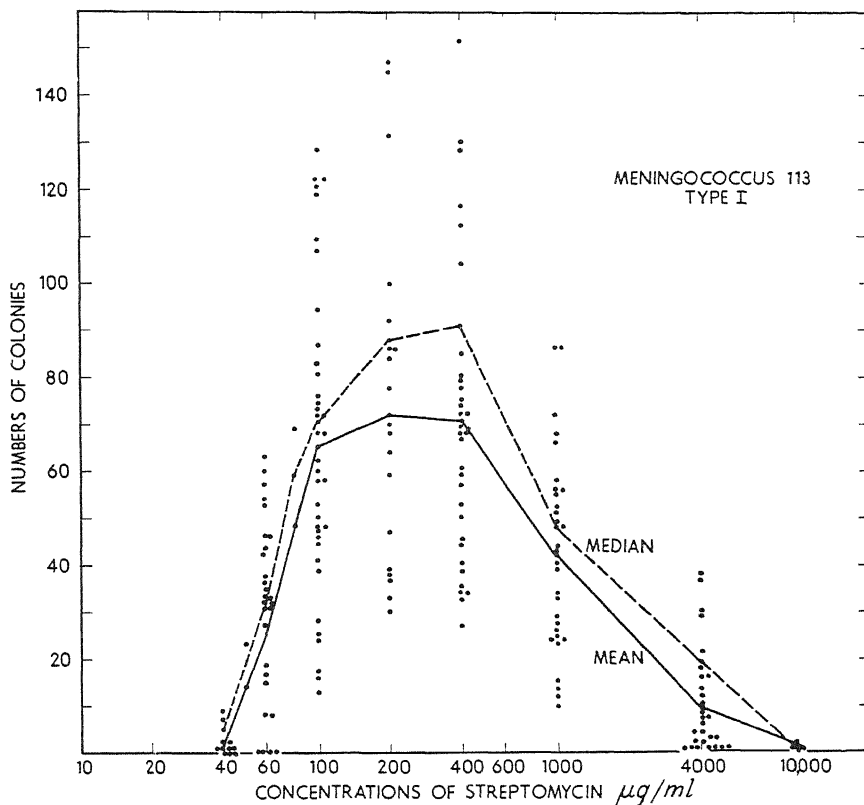


FIG. 3. NUMBERS OF COLONIES OF TYPE B (STREPTOMYCIN-DEPENDENT) VARIANTS DEVELOPING FROM HEAVY SEEDINGS ON GRADED CONCENTRATIONS OF STREPTOMYCIN

Results of 35 experiments with meningococcus 113. The individual inocula contained approximately  $1.0$  to  $2.0 \times 10^{10}$ .

The dependence of type B variants on adequate concentrations of streptomycin for growth was also demonstrated by subculturing them into broth containing graded concentrations of the drug (see figure 4). It will be seen that no growth occurred in the broth containing the low and high concentrations of streptomycin. The optimum range for multiplication in liquid media, therefore, approximated that for solid media.

When a series of plates containing graded concentrations of streptomycin was planted with a pure culture of the B variant in small but equal inocula, the numbers of colonies which developed bore exactly the same relationship to

concentrations of the drug as did the B variants developing from the original inoculations with heavy seedings of the normal, parent strain. These experiments were made as follows: A suspension of B variant was prepared and diluted to a density equal to no. 3 in the McFarland series, which experience has shown to contain about one billion meningococci per ml. This suspension was further diluted a millionfold and a drop (containing 35 to 50 meningococci) planted, by

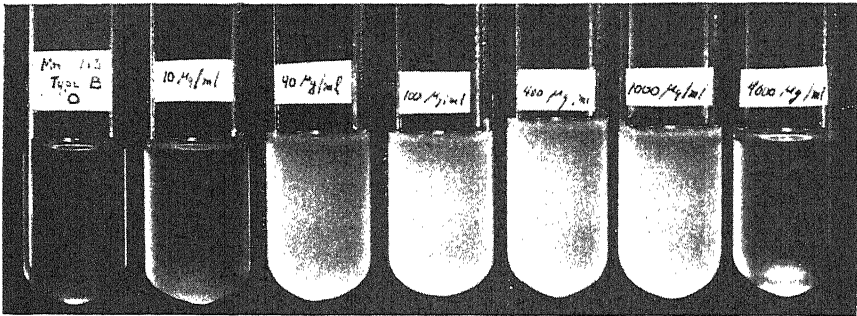


FIG. 4. GROWTH OF TYPE B VARIANTS OF MENINGOCOCCUS IN BROTH CONTAINING GRADED CONCENTRATIONS OF STREPTOMYCIN

Left to right: Tube 1, control. Tubes 2 to 7 contain streptomycin—10, 40, 100, 400, 1,000, 4,000  $\mu\text{g}$  per ml. The tubes were slanted sufficiently to afford a maximum increase in the surface of the broth and incubated for 24 hours.

TABLE 1

*Number and appearance of colonies developing from small, equal inocula of pure culture of type B variant*

STREPTOMYCIN	NUMBER OF COLONIES	DESCRIPTION
$\mu\text{g}$ per ml		
0	0	—
10	0	—
40	3	small, gray
60	8	medium, gray
100	33	medium to large, gray to slightly yellowish
200	35	large, slightly yellowish
400	30	large, yellowish
1,000	25	large, yellowish
4,000	6	small medium, yellowish
10,000	0	—

the method described above, onto a series of plates containing varying concentrations of streptomycin. A portion of the inocula undoubtedly adhered to the beads and was removed with them. The results of a typical experiment are presented in table 1. It shows that the number and appearance of colonies developing on each concentration resemble the number and appearance of type B variants which developed on those concentrations from the heavy seedings made originally with the parent strain. The homogeneity of the culture was estab-

lished by the fact that a number of colonies from each plate transferred onto streptomycin-free and streptomycin-containing agar grew only on the latter.

*Sensitivity of type B colonies to penicillin.* When type B colonies were tested for their sensitivity to penicillin, their growth was inhibited by approximately the same concentrations that inhibited the growth of the normal parent strain from which each variant arose. They appeared, therefore, to be as sensitive as normal meningococci to penicillin. It should be pointed out, however, that the tests could not be made on the same media because of the necessity of providing sufficient streptomycin for the development of type B variants in amounts which were bacteriostatic for the normal strain.

*Reversion of type B variants.* The type B variants continued to exhibit all the characteristics described during repeated subcultivation on streptomycin agar. Their dependence on the drug has been complete except for four instances in which a single colony has developed on streptomycin-free agar. The four exceptions were the only ones to occur among many subcultivations onto streptomycin-free agar. In each instance the colony grew out slowly, but thereafter multiplied readily on streptomycin-free media. They retained all of the properties of meningococci and are regarded as mutations back toward normal. Their reversion to normal was not quite complete, however, for three of them developed no type B variants when planted onto graded concentrations of streptomycin, but only type A. The other reverted strain was able to develop both type A and type B colonies, but the numbers of the former were greater than those produced by its original parent strain. It is clear, therefore, that none of these reverted mutants had regained all of the potentialities of the parent strain from which they were originally derived.

*Effect of inactivated streptomycin.* The type B variants were unable to grow on media containing streptomycin inactivated by hydroxylamine hydrochloride according to the method of Donovan, Rake, and Fried (1946) or by cysteine hydrochloride according to the method of Denkelwater, Cook, and Tishler (1945).

*Experimental infection with type B variants.* The dependence of the type B variants on streptomycin for their multiplication could be demonstrated *in vivo* as well as *in vitro*. When mice were inoculated with mucin suspensions of type B variants, the mice usually survived unless they were treated with streptomycin. An occasional mouse died if very large inocula were used, but meningococci were rarely recovered from its heart's blood, and then only on streptomycin-containing agar.

On the other hand, mice treated with adequate doses of streptomycin usually succumbed to meningococcal sepsis, and type B variants were regularly recovered from cultures of their hearts' blood on streptomycin-containing agar. Although hearts' blood was always planted onto streptomycin-containing and streptomycin-free media, no meningococci ever grew out on the latter.

In table 2 are presented the results of a typical experiment in which mice were inoculated with  $10^8$  or  $10^7$  type B variants. Streptomycin was administered subcutaneously 3 hours after infection in doses of 5, 50, 500, 5,000, and 10,000

$\mu\text{g}$ , the last given in 2 doses of 5,000 each, the second dose  $\frac{1}{2}$  hour after the first. It will be seen that all of the untreated controls survived and that the mortality rose as the dose of streptomycin increased up to the largest dose. One or more

TABLE 2  
*Effect of streptomycin treatment on infection with type B variants*

STREPTOMYCIN TREATMENT 3 HR AFTER INFECTION	APPROXIMATE NUMBERS OF MENINGOCOCCI INOCULATED					
	100,000,000			10,000,000		
	Result	Blood cultures		Result	Blood cultures	
		Strep.-free media	Strep.* media		Strep.-free media	Strep.* media
None	S			S		
	S			S		
	S			S		
	S			S		
5	21†	0	+	S		
	S			S		
	S			S		
	S			S		
50	21	0	+	S		
	90	—	—	S		
	S			S		
	S			S		
500	21	0	+	21	0	+
	21	—	—	21	0	+
	23	0	+	S		
	90	—	—	S		
5,000	21	—	—	23	0	+
	21	—	—	29	0	+
	21	—	—	49		
	27	0	+	S		
10,000 (2 doses of 5,000 ea)	21	0	+	21	0	+
	21	0	+	29		
	21			46		
	S			S		

+ = positive for meningococci; 0 = negative for meningococci; — = not cultured; S = survived.

\* Streptomycin media = media containing 100 micrograms of streptomycin per ml.

† Figures = hours of death.

mice in each group were autopsied and cultures of their hearts' blood made on streptomycin-free and streptomycin-containing agar. In every case meningococci were recovered on the latter but not on the former. The meningococci recovered from the hearts' blood cultures had all of the characteristics of B

variants; that is, they retained their type specificity and their ability to ferment glucose and maltose but required streptomycin for growth.

Unusually large inocula were used in the experiment just described. It has not as yet been possible, however, to produce fatal meningococcal sepsis regularly in mice with inocula smaller than  $10^4$  meningococci even though the animals received multiple injections of streptomycin. The virulence of the type B variants appears, therefore, to be less than that of the type A variants.

#### DISCUSSION

In these experiments two variants have arisen from cultures of meningococcus planted in heavy seedings onto a series of plates containing graded concentrations of streptomycin. It should be emphasized that each experiment was begun with a culture which had never been exposed to streptomycin and that inoculation onto the various concentrations of streptomycin was made at one time. Both variants developed from all of the 18 strains of meningococcus studied. They were gram-negative diplococci which retained the characteristic sugar fermentation and type specificity of the parent strains from which they arose. Both variants were highly resistant to streptomycin.

One variant, designated type A, grew in large, yellowish colonies which appeared in approximately equal numbers on all concentrations of the drug although the numbers varied considerably from strain to strain. Its resistance to streptomycin was demonstrated *in vivo* as well as *in vitro*, for it produced infection in mice which proved uniformly fatal in spite of the administration of maximal doses of streptomycin tolerated by the mice.

The incidence of the other variant, designated type B, as well as the size and color of its colonies depended on the concentrations of streptomycin onto which the original seedings were planted. Nevertheless, all of the B variants derived from any strain were found to be genetically alike.

The striking characteristic of this variant was its dependence on streptomycin for multiplication on solid and in liquid media and in the body of an animal host. The animal experiments indicated that this variant was nonvirulent for mice unless the animals were treated with adequate doses of streptomycin and that the dependence on streptomycin for growth persisted during and after multiplication within the body of the infected animal.

It is impossible at the present time to be certain whether the substance required for the growth of the type B variant is streptomycin itself or some impurity which has been present in all of the preparations we have used. These numbered 25 and were obtained from seven manufacturers. Two of the preparations were described as being of an especially high degree of purity. It should be noted that streptomycin inactivated by hydroxylamine or by cysteine failed to support growth of the type B variants. This aspect of the problem is under investigation.

The origin of these variants is difficult to explain unless one assumes that they both arise by current mutation; i.e., from mutants which are constantly appearing in the original bacterial population of the parent strain. The type A

variants developed from any given strain with about equal frequency on all concentrations of streptomycin, although the frequency varied from strain to strain.

The incidence of the type A variants from most strains was estimated to average 1 to 3 in  $10^{10}$  of original bacterial population. One strain produced about 3 to 30 in  $10^{10}$ .

The maximum incidence of the type B variants varied from 2 to 15 per billion meningococci in the parent culture.

When the type B variants were first observed, they were thought to arise by mutation which was induced by streptomycin. Subsequent observations have failed to support this hypothesis and have tended instead to indicate that, like the A variants, they, too, originated from mutations which were occurring regularly in the parent bacterial population.

The fact that they appeared only on the high concentrations and only in greatest numbers within a certain range was explained by the demonstration that this range of concentrations was optimal for their development. Pure cultures of the B variants developed colonies on each concentration of streptomycin in the same relative numbers as did heavy seedings of the parent strain from which they arose. The greater proportion of them were able to reproduce only on certain concentrations; above and below that optimal range few or none developed. In other words, the type B variants developed approximately the same number of colonies on each of a series of concentrations whether they were planted in pure culture or together with myriads of normal, streptomycin-sensitive meningococci. This observation seems to indicate that the streptomycin requirement of the type B variants for their multiplication is quantitative as well as qualitative.

The variation in size and color of colony of the B mutants can only be attributed to the direct effect of streptomycin on the physiology of the microorganisms. Benham (1947) found that streptomycin increased the oxygen uptake of a normal strain of typhoid bacilli but not of a resistant one unless high concentrations of the drug were used.

Several studies on the development of streptomycin resistance have appeared: Chandler and Schoenbach (1947) for staphylococcus, streptococcus, and pneumococcus; Hamre, Rake, and Donovan (1946) for *Klebsiella*; and Klein and Kimmelman (1946a, 1946b). Alexander and Leidy (1947), using a technique similar to ours, isolated streptomycin-resistant variants from *Hemophilus influenzae* and estimated their incidence as 1 in 1.1 billion to 1 in 13.8 billion members of the original bacterial population. As none of these authors mentions dependence on streptomycin as a characteristic of the resistant strains, one must conclude that they were dealing with resistant variants analogous to the type A variants herein described. It is quite certain that the streptomycin-resistant gonococci and meningococci reported earlier by Miller and Bohnhoff (1946a) were type A variants.

Hall and Spink (1947) describe a strain of *Brucella* which became highly resistant to streptomycin. This strain was recovered from the blood stream of a

patient with *Brucella* endocarditis and had apparently developed a considerable degree of resistance *in vivo*. After it had become highly resistant, it produced two types of colonies, a large one, which grew rapidly, and a small one, which grew slowly. Although this latter variant was able to grow on streptomycin-free agar, it grew better on media containing 50 to 100  $\mu\text{g}$  of streptomycin per ml. It is possible that this second type of colony is similar to our B variant.

It should be noted that Welch, Price, and Randall (1946) were able to demonstrate large numbers of viable typhoid bacilli in broth cultures containing streptomycin in concentrations greater than the minimum which inhibited growth. They also found that the mortality rate of mice infected with typhoid bacilli was increased by treatment with small doses of streptomycin (0.05 to 1.0  $\mu\text{g}$ ). The mortality rate, however, was decreased when larger doses were administered.

For a comprehensive discussion of the general problem of bacterial mutation, the reader is referred to the recent review by Luria (1947).

Studies on the growth requirements of mutants isolated from cultures of *Escherichia coli* after treatment with bacteriophage (Anderson, 1944; Luria and Delbrück, 1943; Luria, 1945) or X-ray (Tatum, 1945; Gray and Tatum, 1944) have demonstrated a variety of deficiencies in their metabolic processes. Similar observations have been made on mutants induced in *Neurospora* by X-ray (Beadle, 1945).

Emerson (1944) has described a mutant of *Neurospora* which required sulfanilamide for growth and for which *para*-aminobenzoic acid was toxic. This variant appeared in his cultures only once.<sup>\*</sup> The type B variants of meningococcus, on the other hand, developed regularly from all of 18 strains which included types I, II, and II alpha, some of which strains had recently been isolated from cases of epidemic meningitis and some from carriers, but others were old stock strains which had been under cultivation in the laboratory for many years.

#### ACKNOWLEDGMENTS

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#### SUMMARY

Two streptomycin-resistant variants developed from each of 18 strains of meningococcus, including types I, II, and II alpha, when heavy seedings were planted onto a series of plates containing streptomycin in concentrations varying from 40 to 10,000  $\mu\text{g}$  per ml. One variant, designated type A, appeared in small and approximately equal numbers on all concentrations. It grew in large yellowish colonies on streptomycin-free and streptomycin-containing media. It retained the original virulence for mice possessed by its parent strain.

The other variant, designated type B, appeared in greatest numbers on concentrations between 100 and 400  $\mu\text{g}$  per ml, the concentrations optimal for its

<sup>\*</sup> Personal communication to authors.

multiplication. Its colonies varied in size and color depending upon the concentrations of streptomycin on which they developed. They were small and gray on concentrations of less than 100  $\mu\text{g}$  per ml and larger and slightly yellowish on concentrations of 200  $\mu\text{g}$  or more per ml. Nevertheless, the type B variants from any strain were found to be genetically identical and the differences in their colonial appearance to be determined by the concentration of streptomycin on which they grew.

The type B variants were dependent on streptomycin for multiplication *in vitro* and *in vivo*. They were nonviable on media containing concentrations of less than 5  $\mu\text{g}$  per ml and grew best on 100 to 400  $\mu\text{g}$  per ml. They were nonvirulent for mice, unless the mice received streptomycin. In mice treated with streptomycin, they produced a fatal meningococcal sepsis and were recovered from the hearts' blood provided the cultures were made on streptomycin-containing media.

Both variants retained the characteristic sugar fermentations of meningococci and the type specificity of the parent strains from which they arose. Both variants are presumed to arise from mutants which are constantly appearing in the bacterial population of the parent strain.

Whether the substance required by the B variants for their multiplication is streptomycin itself or some impurity has not yet been determined. These variants developed on all of 25 preparations of streptomycin obtained from 7 manufacturers. They failed to develop on streptomycin inactivated by hydroxylamine or by cysteine.

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# ISOLATION AND CYTOLOGICAL STUDY OF A FREE-LIVING SPIROCHETE

M. T. DYAR

*Department of Microbiology, University of Washington, Seattle 5, Washington, and Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York*

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The genus *Spirochaeta* includes the large, free-living, spiral microorganisms of the type described by Ehrenberg (1833) as *Spirochaeta plicatilis*. Zuelzer's (1910, 1912) careful studies of *Spirochaeta plicatilis* provide the most detailed description of the type species of the genus *Spirochaeta*. Zuelzer found this spirochete growing in both fresh- and salt-water enrichment cultures in close association with *Beggiatoa*, *Oscillatoria*, and other microorganisms. She described it as a flexible, spiral, blunt-ended organism 100 to 200, rarely 500, microns in length,  $\frac{1}{2}$  to  $\frac{3}{4}$  microns in diameter, and with regular, steep spirals having a wave length of 2 microns. The cells contained volutin granules. Motility was by screwlike, vibrating, flexing movements and, on solid surfaces, by a creeping movement. Multiplication was by simple or multiple transverse division. Zuelzer did not recognize a definite cell membrane, but some other early workers, as reviewed by Bosanquet (1911), described a definite "periplastic sheath." Zuelzer considered the most distinguishing characteristic of *Spirochaeta plicatilis* to be a straight, elastic, axial filament around which the protoplasm was wound and which she observed in both living and stained cells.

Other members of this genus have been described by Cantacuzene (1910), Dobell (1912), Zuelzer (1912, 1923), Pettit (1928), Gardner (1930, 1932), and other investigators. Unfortunately, all of the descriptions are based on preparations made from transient, mixed cultures, for no member of the family *Spirochaetaceae*, which includes *Spirochaeta*, *Saprospira*, and *Cristispira* (Bergey, 1947), has ever been reported grown in pure culture. Therefore, it has been impossible to make repeated and controlled studies on a given species, and, as a result, considerable confusion exists concerning the relationships of these microorganisms.

We have succeeded in isolating a species of free-living *Spirochaeta*, believed to be *Spirochaeta plicatilis*, and have maintained it in pure culture for almost three years. This paper deals with the isolation and with the cultural characteristics and cytology of this spirochete.

## ISOLATION

Enrichment cultures of the spirochete were obtained from infusions of decaying leaves from a hydrogen sulfide spring. It was found that spirochetes, in association with *Oscillatoria*, *Beggiatoa*, and many other species of algae, bacteria, and protozoa from the enrichment cultures, grew sparsely on the surface of dilute leaf decoction agar plates. Microscopic observation of the plates revealed the

large, spiral, flexible spirochetes among the other microorganisms. Occasionally a group of spirochetes grew away from most of the other growth on a plate. Such areas were marked and a speck of agar was carefully transferred to another plate. Repeated transfers on the decayed leaf medium eliminated many, but never all, of the contaminating species which were apparently contributing necessary growth factors. Addition of a few drops of blood to the leaf agar enabled the spirochetes to outgrow the last remaining contaminants. The pure culture of spirochetes obtained in this way was unable to grow on the leaf agar but grew well on the surface of medium containing 5 to 10 per cent sterile red blood cells and 1 to 1.5 per cent agar.

Although only one strain has been isolated, spirochetes very similar in general appearance have frequently been observed in enrichment cultures from various ponds and springs in both Washington and New York states.

#### CULTURAL CHARACTERISTICS

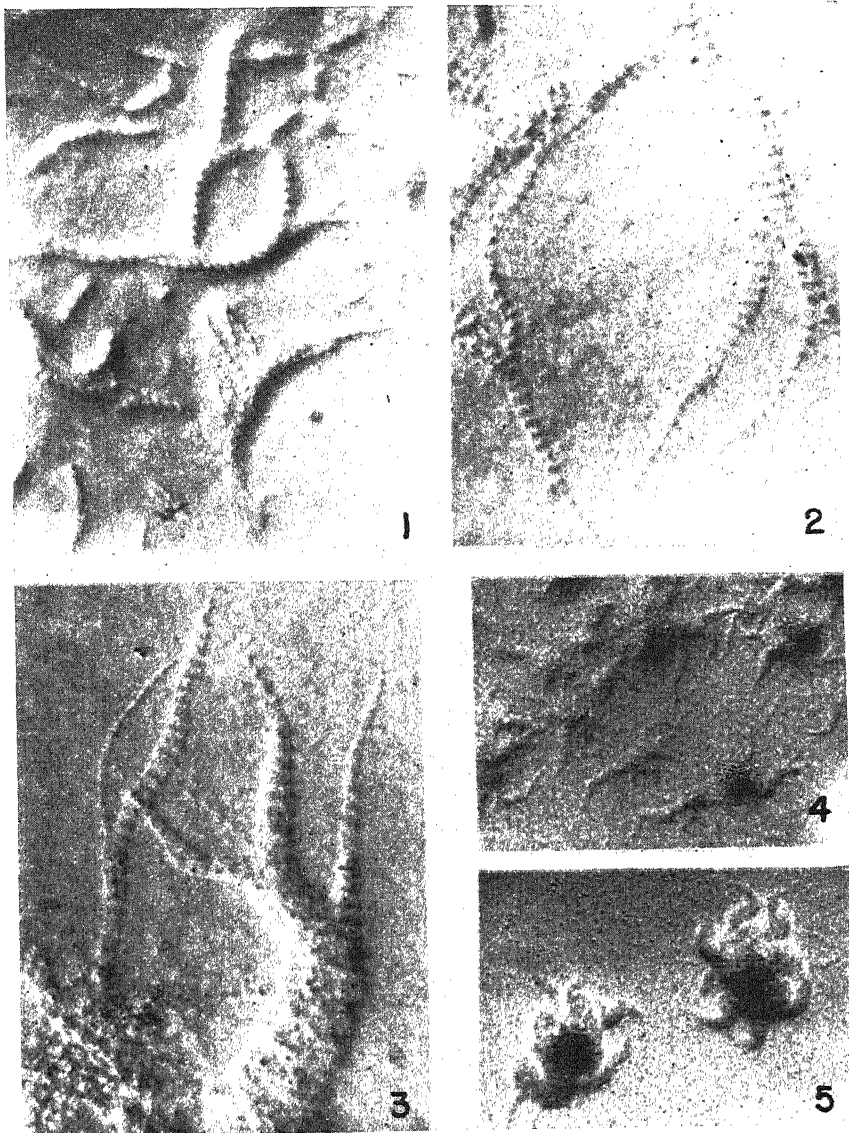
The spirochetes grow poorly or not at all on the serum fraction of blood but multiply readily on the red cell fraction, even when the red cells have been repeatedly washed. They have not been cultivated on any ordinary laboratory media, in liquid media, or on heat-sterilized blood. Hydrogen sulfide, although present in the spring from which the spirochetes were isolated, is apparently unnecessary for growth. The spirochetes are aerobic, grow in a pH range of 6 to 9 and in a temperature range of 15 to 34 C; the optimum temperature is about 26 C. Stock cultures are more successfully kept in small flasks than on slants and are transferred by washing off the growth with sterile water. Cultures remain viable for about a month at room temperature but not when stored for a similar period of time at refrigerator temperature.

The spirochetes ordinarily grow slowly on the surface of the medium as a thin, spreading film just visible to the eye. Growth is apparent after one to several days. Examination of a plate culture with the low powers of the microscope reveals the cells scattered on the surface as shown in figure 1 or often lying side by side with their spirals closely fitted together. Groups of such closely associated cells may advance at the edges of the diffuse growth in flamelike projections as shown in figures 2 and 3. They may occasionally pile up into discrete colonies as shown in figures 4 and 5, but this behavior is very uncommon.

Motility on an agar surface is accomplished by a slow, forward, screwlike rotation of the cells, and sometimes a trail on the agar can be seen behind a moving spirochete like the track behind a snail. The motion of spirochetes suspended in water is both screwlike and slowly but constantly flexing, and on a moist surface one end of a cell may remain attached while the other oscillates back and forth. Cells may congregate in a droplet of moisture on a plate and revolve around and around in it. Such a coiled cell, removed by making a cover slip impression, is shown in figure 12.

#### MORPHOLOGY AND CYTOLOGY

The spirochetes have been studied at different ages by means of light- and dark-field observation of living cells and by various staining procedures. The



FIGS. 1 to 5. Spirochetes growing on the surface of blood agar; photographed with side illumination.

FIG. 1. Typical growth with irregularly scattered cells.  $\times 250$ .

FIGS. 2 AND 3. Groups of cells at edges of diffuse growth.  $\times 250$ .

FIGS. 4 AND 5. Cells aggregated into colonies; this type of growth is not common.  $\times 50$ .

author is grateful to Dr. R. F. Baker of the RCA Laboratories and to Dr. Georges Knaysi for electron photomicrographs which have greatly aided the cytological study. The electron micrographs of cells grown for 8 to 10 days on blood agar are shown in figures 7 to 11. The author is grateful also to Dr. Oscar W. Richards

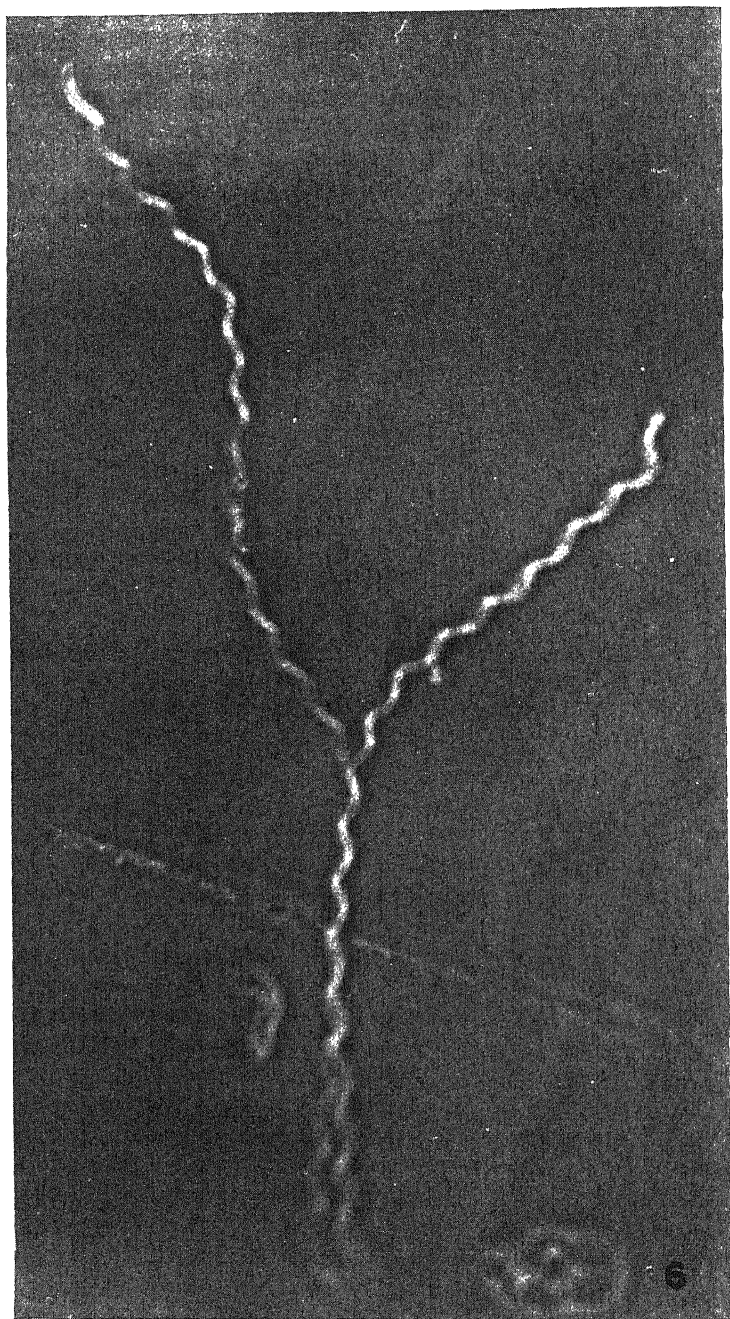


FIG. 6. Living spirochetes photographed with the bright contrast phase microscope (American Optical Company).  $\times 1,800$ .

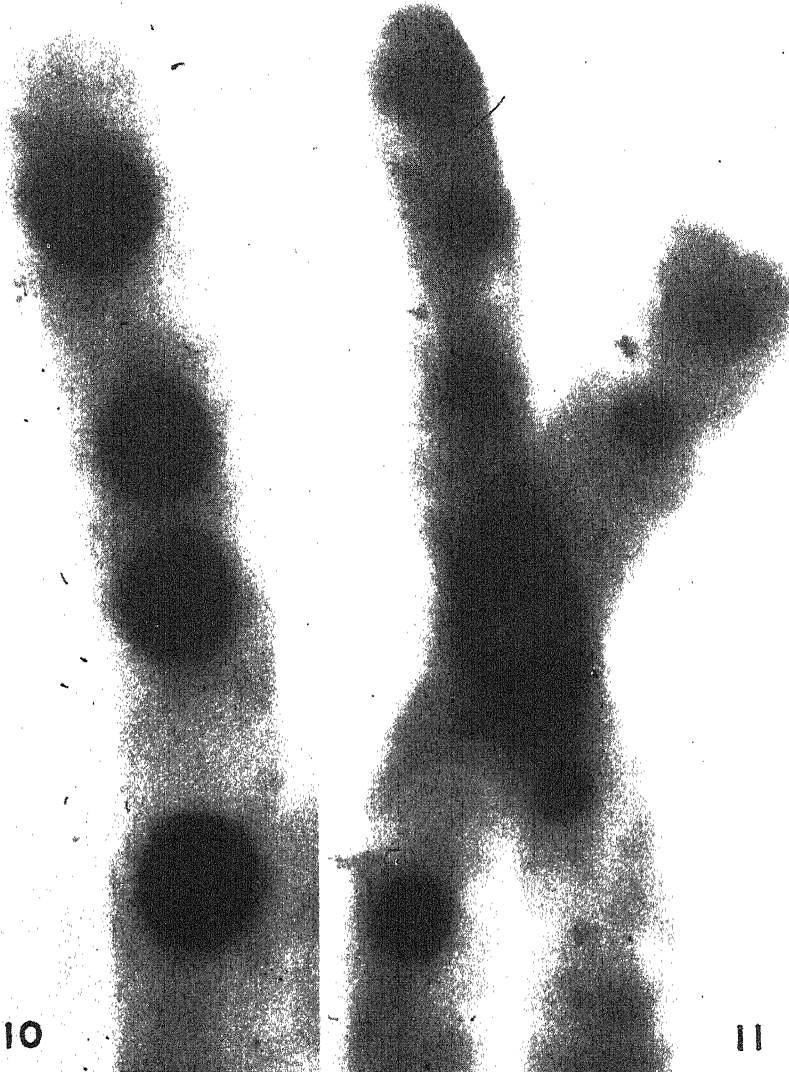
of the American Optical Company for allowing her to examine the spirochetes with the phase microscope and for taking the bright contrast phase photomicrograph of living cells which is shown in figure 6.



FIGS. 7, 8, AND 9. Spirochetes photographed with the 50 kv RCA electron microscope. Most of the granules are volutin. The cell shown in figure 9 has fragmented.  $\times 18,000$ .

The living spirochetes are flexible, regular spirals with a diameter of 0.8 to 1.2 microns and a length ranging from 1 to 2 wave lengths to several hundred microns. Individuals 400 microns long are fairly common, and even longer ones

occur occasionally. That the spirochetes are spiral instead of wavy can be seen by focusing and is apparent on the phase photomicrograph. In young spirochetes the wave length varies from 3 to 6.5 microns depending on how tightly



FIGS. 10 AND 11. Spirochetes photographed with the 50 kv RCA electron microscope.  $\times 36,000$ .

the spiral is coiled, and the spiral amplitude is about 2 microns. However, these dimensions become more inconstant in old cells, which may either unwind into loose, irregular spirals or straighten out almost completely. Likewise, cells dried and fixed usually become straight.



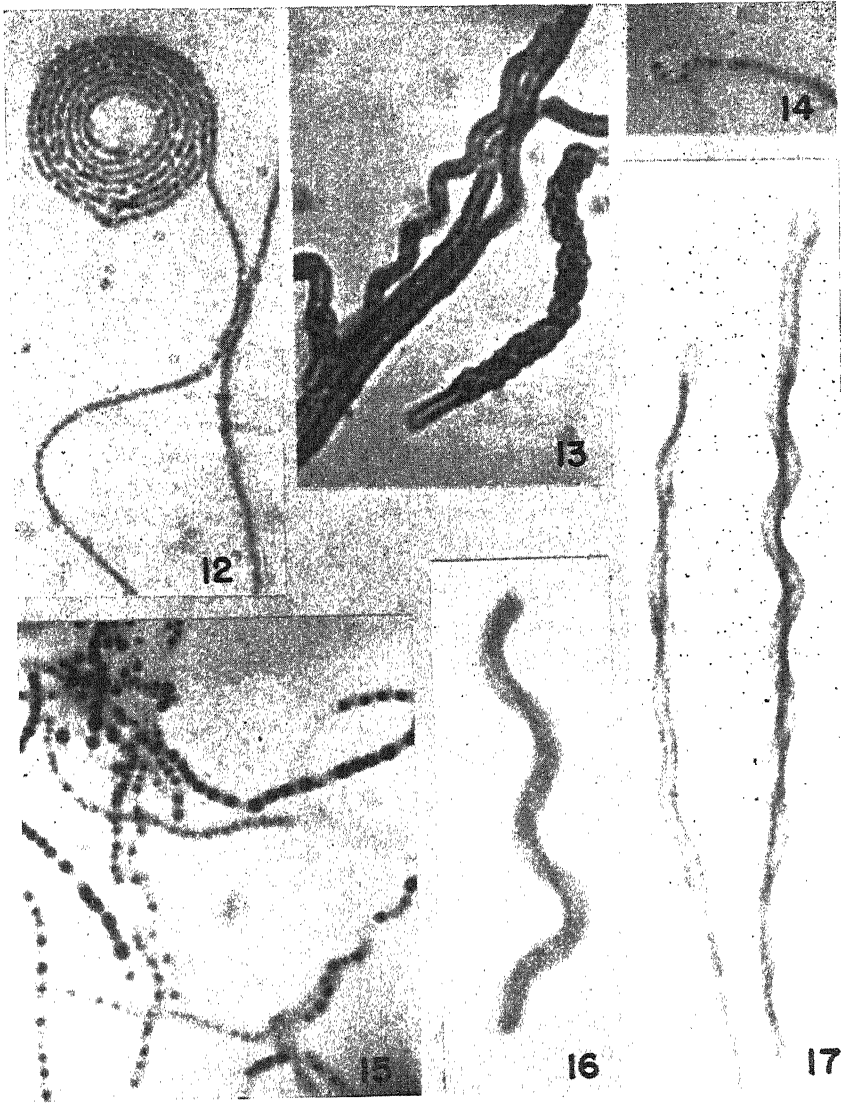


FIG. 12. Coiled spirochete removed from a plate by making a cover slip impression; fixed and stained with Giemsa's solution.  $\times 1,350$ .

FIG. 13. Spirochetes stained with a cell wall stain. One cell has retained the spiral form and another shows the cell membrane distended by volutin granules.  $\times 2,250$ .

FIG. 14. Spirochete from an old culture showing a swelling at one end.  $\times 1,350$ .

FIG. 15. Spirochetes stained with methylene blue to demonstrate volutin inclusion.  $\times 2,250$ .

FIG. 16. Cell of a large *Spirillum* stained with a flagella stain to illustrate the appearance of an "axial filament."  $\times 2,500$ .

FIG. 17. Spirochete cells stained with Giemsa's solution to demonstrate the "axial filament."  $\times 2,500$ .

In old cultures the cells sometimes form swellings, as in figure 14, similar to the plasmolysis figures reported by Dobell (1912) for *Saprospira flexuosa*. Such cells may still be motile.

Many refractive granules are apparent in spirochetes examined by either light- or dark-field illumination. The inclusions are of at least two types: volutin, which has frequently been reported to occur in free-living spirochetes, and fat. We have followed the inclusions at intervals in cultures from several days to more than a month old. The volutin inclusions are identified by their intense and metachromatic staining with methylene blue and by their solubility in hot water and in 0.02 per cent  $\text{NaHCO}_3$ . Volutin granules in cells stained with methylene blue show clearly in figure 15. These inclusions are arranged either regularly or irregularly in the cell and range in size from small specks to relatively immense bodies occupying the entire diameter of the cell. The larger ones are often compressed into a rectangular shape and may stain more intensely around their border with basic dyes, suggesting a vacuolar rather than granular character. They are present in greatest abundance and size in cultures a few days to a week old but persist in many cells even in month-old cultures. Most of the inclusions seen in the electron photomicrographs are volutin. Granules of a volutin nature frequently persist after old cells have disintegrated; there is no evidence that these represent anything but degenerated cells.

The fat inclusions are identified by deep staining with Sudan black B when cells are suspended in a saturated ethylene glycol solution of this dye. Cells 4 to 7 days old contain many fat granules, and some cells from month-old cultures still contain small ones. The volutin and fat inclusions are distinct from each other, as may be observed when Sudan black B solution is allowed to run under a cover slip onto a film already stained with methylene blue.

The electron photomicrographs show the presence also of inclusions too minute to be resolved by the light microscope. The cytoplasm stains with basic dyes and is gram-negative.

Spirochetes of various ages have been hydrolyzed with  $\text{N HCl}$  at 60 C for 10 minutes and stained both by the Feulgen method and with Giemsa's solution. By either method the cells appear somewhat granulated or stippled, this effect being more apparent when they are examined wet than when in oil. A few Feulgen-positive granules were observed which may be nuclei, but more work should be done before this is certain.

A cell membrane, which does not stain readily with Giemsa's solution or with basic dyes, may be demonstrated with Dyar's (1947) cell wall stain. The membrane in the living cell is, of course, flexible and in a fixed, stained cell shrinks in close to the cytoplasm even when examined wet. The cell wall stain shown in figure 13 shows the membrane shrunk except where it is distended by large volutin granules, presenting much the appearance of a tight rubber skin stretched over a string of beads. Delicate, refractive cross walls have been seen in a few living cells examined by dark-field illumination, and, likewise, occasional individuals when stained by the cell wall method are seen to consist of shorter cells, each with a complete membrane around it. The spirochetes with cross

walls seen by these methods are relatively few and rather clearly represent a stage of multiple transverse division.

Dried or fixed cells sometimes fragment into regular segments each about half a wave length long as shown in the electron photomicrograph in figure 9. However, the prominent, regular cross striations which are seen in specimens of *Saprospira* and *Cristispira* stained with Giemsa's stain or with basic dyes and which give these organisms their characteristic "chambered" appearance are not evident in stained specimens of this spirochete nor in the electron photomicrographs. Nor are regular cross striations detectable in living cells by either ordinary light- or dark-field illumination.

However, examination with the phase microscope (Bennett, Jupnik, Osterberg, and Richards, 1946) reveals fine, delicate cross septae occurring throughout the length of living cells. In young cells the septae are very striking for their clarity and regular spacing at half turns of the spiral in all cells. In old cells the cross walls are also clearly present, although they may be somewhat less distinct and more irregular in spacing. The spirochetes are clearly not single, long spiral cells but multicellular spiral filaments.

No flagella have been demonstrated with Leifson's (1930) flagella stain, nor do the electron photomicrographs give any evidence of flagella.

An investigation of the "axial filament" seemed especially important because of the prominence it has been given in characterizing *Spirochaeta*. Zuelzer (1910) was the first to describe this structure as a straight, elastic filament around which the protoplasm was wound. Some other investigators such as Bach (1921) and Gardner (1930) have confirmed its presence. On the other hand, Dobell (1912), although he accepted the concept of an axial filament, was unable to observe it in the several species of *Spirochaeta* that he studied; and Noguchi (1928) was unable to demonstrate it in a spirochete from the slime of an icebox drain. Certainly not every investigator will admit its existence.

In this spirochete, an "axial filament" has been demonstrated, as shown in figure 17, but only rarely in preparations stained overnight in Giemsa's solution and by no other method. It has been apparent only in preparations where the cells are heavily outlined with stain as a result of the long staining time, and then only in cells that have dried in the spiral form, never in straight individuals even though they be adjacent on the slide. The "axial filament" appears to be continuous with the heavily stained cell outline. The outline ordinarily is apparent just at the cell borders where one is looking through the greatest thickness of stain. However, in a spiral cell the places where the cell spirals around also present a greater thickness, resulting in the appearance of a heavily stained filament lying in the axis of the spiral. Giemsa staining does not show such a structure in spiral cells of *Spirillum*, probably because it is masked by the entire cell's being very intensely stained; but, indeed, the appearance of an axial filament can be produced in a large species of *Spirillum*, as shown in figure 16, by the use of Leifson's flagella stain, a procedure which precipitates stain on the cell. In this *Spirillum* no such structure is apparent by any other procedure.

Therefore, an axial filament is thought to be an artifact resulting from the appearance of stain deeply outlining the spiral form. The light red cell wall stain does not seem to be intense enough to duplicate this effect.

Neither examination of living spirochetes with the phase microscope nor the electron photomicrographs show any evidence of an axial filament in this spirochete. Likewise, electron photomicrographs of *Treponema pallidum* taken by Morton and Anderson (1942), of three species of *Treponema* taken by Mudd, Polevitzky, and Anderson (1943), and of *Borrelia novyi* taken by Lofgren and Soule (1945) show no axial filament in these organisms.

#### DISCUSSION

It is apparent that there is a very close resemblance between this *Spirochaeta* and *Spirochaeta plicatilis* in regard to natural habitat, cell form and size, type of cell inclusions, division, and motility. The dimensions do not coincide exactly with those reported by Zuelzer for *Spirochaeta plicatilis*; however, we do not believe that the inconsistency represents a real difference, because our cells were measured alive and hers presumably were measured after fixing and staining. Actually, a stained cell such as the spiral one in figure 13 of this paper and the ones shown in table 1 in Zuelzer's (1912) paper are almost identical in respect to diameter, wave length, and spiral amplitude. Furthermore, although we consider the "axial filament" as an artifact, the appearance of such a structure has been obtained with Giemsa's solution, one of the procedures that Zuelzer used to demonstrate it.

There seems little question that the free-living *Spirochaeta* which we have isolated is identical with the type species, *Spirochaeta plicatilis*.

The presence of an axial filament and the absence of a distinct periplast membrane and of prominent cross striations in stained specimens are three important characteristics used to differentiate *Spirochaeta* from *Saprospira* and *Cristispira* (Bergey, 1947); *Cristispira* is further separated on the basis of its crista and parasitic habitat. However, these criteria surely need reconsideration because in the present study we have thrown considerable doubt on the reality of the axial filament. Furthermore, although the membrane and cross septae of this spirochete are not readily apparent by usual procedures, a membrane is clearly demonstrable by a cell wall stain, and regular cross septae by observation of living cells with the phase microscope. We are not convinced, on the basis of the present knowledge, that *Spirochaeta* and *Saprospira*, as described by Gross (1911), should be considered distinct genera.

We believe that a truly satisfactory relationship among the *Spirochaetaceae* can be established only when more representatives of this group have been isolated and studied under reproducible and comparable conditions.

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# THE ENTEROCOCCI: WITH SPECIAL REFERENCE TO THEIR ASSOCIATION WITH HUMAN DISEASE

ALICE C. EVANS AND ALICE L. CHINN

*National Institute of Health, Bethesda, Maryland*

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During the past 15 years, when beta hemolytic streptococci of Lancefield's (1933) groups A and C were subjects of investigation in this laboratory, strains of group D were acquired from time to time and were included in the comparative studies. In this paper they will be called enterococci, a term first used by Thiercelin (1899).

Because the enterococci have been found to be resistant to the sulfonamides and to penicillin, and because the doctor wants to know as early as possible in the course of a disease what kind of treatment is indicated, the importance of the recognition of the enterococci has increased in recent years.

Sherman and his collaborators (1931, 1937, 1943) studied the enterococci extensively, and Sherman (1937) published a review of the literature. Their studies included few pathogenic strains, and the review did not include a full consideration of the incidence of enterococci in human disease.

## THE HARDINESS OF ENTEROCOCCI

Sherman (1938) reported other distinguishing characteristics of the enterococci in addition to precipitation in serum of group D—namely, the ability to grow at 10 C and 45 C; survival at 60 C for 30 minutes; and tolerance for inhibitory substances, as shown by ability to grow in the presence of 6.5 per cent sodium chloride, 0.1 per cent methylene blue, or 40 per cent bile.

The tolerance of the enterococci for inhibitory substances is of practical importance. A high degree of resistance to the sulfonamides was reported by Bliss and Long (1937), Long and Bliss (1938), Neter (1938, 1940), Helmholz (1937, 1941), Francis (1941), Rantz and Kirby (1943), and MacNeal and Blevins (1945).

In his original paper on the inhibitory action of penicillin on many species of bacteria, Fleming (1929) noted that enterococci were resistant to it. This finding was confirmed by later investigators (Bornstein, 1940; Heilman and Herrell, 1942; McKee and Rake, 1942; Watson, 1944; White and Sherman, 1944; MacNeal and Blevins, 1945).

## THE SOURCES OF THE STRAINS OF OUR COLLECTION

Our collection contained 23 strains from human pathologic sources and 11 strains from other sources, included for comparison. Three of the latter were from the stools of normal human subjects; one was from a normal human throat; two were from different pooled lots of human plasma; one was from an unknown human source; one was isolated from milk powder and one from pasteurized

milk; one was from the diseased lung of a dog; one, received from the American Type Culture Collection, had a history of having been isolated from pus from a horse with strangles. The authenticity of this origin is questionable, because the strain came indirectly from the original investigator and was labeled "*S. equi*."

Some of the strains listed in table 1 are duplicates from the same patient. It appears that more than one of the strains (nos. 1188, 1308, 1355, and 1357) that were received from Dr. Sherman may have been isolated at different times from the same patient, because, according to Sherman, Stark, and Mauer (1937), *S. zymogenes* was isolated on various occasions from one subject. Strains 1332 and 1333 were originally the same strain, one of the branches having had a history of undergoing variation in its ability to hemolyze blood.

#### THE CHARACTERISTICS OF THE ENTEROCOCCI

The strains included in table 1 belonged to group D, according to Lancefield's precipitin test; all grew at 10 C and 45 C; all grew in media containing 6.5 per cent NaCl and in media having an initial pH value of 9.6; all tested strains (31) grew in media containing 40 per cent bile; all hydrolyzed esculin; none hydrolyzed starch; all attained a final pH of 4.4 or lower in glucose broth, except one (no. 945) which produced a final pH of 4.6; all strains fermented maltose; of 27 strains tested, all fermented salicin; of 29 strains tested, all fermented trehalose. None of 25 tested strains fermented dulcitol or inulin.

The characteristics which were common to all strains are omitted from table 2, which includes those reactions which showed interesting differences between strains. Omitted from the table are the following reactions: hydrolysis of sodium hippurate, production of ammonia in 4 per cent peptone, and virulence for mice. These reactions appeared not to be correlated with significant characteristics.

Though not considered in table 2, a general statement in regard to the virulence of the enterococci for mice may be of interest. Mice were injected intraperitoneally with broth cultures, which had been inoculated very lightly by platinum needle and incubated for about 11 hours. Two strains killed mice in  $10^{-2}$  dilution; 22 killed in  $10^{-1}$  dilution but not in higher dilutions; 9 strains failed to kill in the  $10^{-1}$  dilution. According to these results, the virulence of enterococci for mice is low as compared with the virulence of many strains of streptococci of groups A and C.

Uncorrelated with characteristics which appeared to be significant for classification purposes are the following, listed in table 2: type of hemolysis; liquefaction of gelatin; sensitivity to bacteriophage D<sub>2</sub>-1188; production of acid from lactose, arabinose, and raffinose. The characteristics of distinction were found to be agglutinative reactions, sensitivity to phage D-693, growth in milk containing 0.1 per cent methylene blue, survival at 60 C for 30 minutes, and production of acid from sucrose, mannitol, and sorbitol. In the study of a larger series of strains, the production of acid from glycerol might be found to be of some significance.



TABLE 1

*Histories of streptococcal strains of group D*

NIH NO.	PREVIOUS INVESTIGATORS	PREVIOUS DESIGNATION	SOURCE		
			Host	Material	Disease
693	Kendrick and Hol- lon; Abbott Lab- oratories	<i>S. fecalis</i> no. 31 1900.89	Human	Feces	Intestinal hemor- rhages
696	The Lilly Research Laboratories	<i>S. hemolyticus</i> no. 1527	Human	Pus	Empyema
702	Allen-Sandlin Lab- oratories		Human		Sore throat
894	Graham	18297	Canine	Lung	
912		S 56	Human	Tooth	Infected
913	Thompson and Me- grail	S 112	Human	Feces	None
914		S 115	Human	Feces	None
945	Dr. John S. Buckley; Am. Type Culture Collection, Cata- logue of Cultures, 1927	A.M.S 6; 827	Equine?	Pus?	Strangles?*
977	Torrey and Montu	Intestinal strep- tococcus no. 11	Human	Feces	Chronic ulcera- tive colitis
978		Intestinal strep- tococcus no. 13	Human	Lesion	Chronic ulcera- tive colitis
979		Intestinal strep- tococcus no. 24	Human	Feces	Chronic ulcera- tive colitis
980		Intestinal strep- tococcus no. 14	Human	Feces	None
1121		L-14	Human		Osteomyelitis
1122		L-50	Human	?	?
1130		32	Human	Pus	Peritoneal ab- scess
1131	Lederle Labora- tories	198	Human	Heart blood	Septicemia fol- lowing small- pox vaccina- tion
1132		291	Human	Pus	Ear infection fol- lowing measles
1181		Hilligest	Human		Meningitis fol- lowing mas- toiditis
1187	Farrell	R-36	Human		Normal throat
1188	Sherman, Stark, and Mauer		Human	Feces	Diarrhea
1275	Saunders; Torrey and Montu	66	Human		Gastric ulcer car- cinoma
1276		83	Human		Gastric ulcer
1278		140	Human		Gastric ulcer car- cinoma

TABLE 1—*Continued*

NH NO.	PREVIOUS INVESTIGATORS	PREVIOUS DESIGNATION	SOURCE		
			Host	Material	Disease
1308	Sherman, Stark, and Mauer	<i>S. zymogenes</i> no. 1	Human	Feces	Intestinal dis- order
1309	Sherman and Wing	<i>S. durans</i> Wing 3		Milk powder	
1332	Dr. E. C. Rosenow	257.21†	Human		Endocarditis
1333		257.18†			
1355	Sherman, Stark, and Mauer	26C <sub>1</sub>	Human		Intestinal dis- order
1357		36C <sub>2</sub>	Human		
1359		132B		Pasteurized milk	
1531		Varn	Human		Peritonitis
1574		Rigens	Human	Pus	Otitis media
1588		3365	Human	Pooled plasma	
1600		31A	Human	Pooled plasma	

\* The authenticity of the source of strain 945 is questioned (see the text).

† Strains 1332 and 1333 were from the same patient.

#### TESTS FOR THE DIFFERENTIATION OF ENTEROCOCCAL SPECIES

According to the key in the fifth edition of *Bergey's Manual* (1939) liquefaction of gelatin and hemolysis are considered to be distinctive characters, on which the differentiation of enterococcal species is based. Sherman, Stark, and Mauer (1937) mentioned the "thin and shaky boundaries" which separate the "supposed" enterococcal species, but Sherman (1938) recognized 3 species which he differentiated on the basis of the two characteristics mentioned above.

Durand and Dufourt (1923) reported that they found a precise correlation between liquefaction of gelatin and agglutinative reactions. According to other investigators, however, liquefaction of gelatin is an unstable property of no significance in classification. Houston (1934) noted that the action of bacteriophage may alter the gelatin-liquefying property. Elser and Thomas (1936) found gelatin-liquefying strains of enterococci which agreed well with nonliquefying strains in cultural and biochemical properties. Wheeler and Foley (1943) stated that biologic characteristics of enterococci could not be correlated with serologic type.

Lack of correlation between liquefaction of gelatin and significant characteristics may be noted in table 2. For example, strains 1278 and 1600 gave almost identical reactions in all tests excepting that for liquefaction of gelatin.

Gordon (1922) found that hemolytic and nonhemolytic strains of enterococci behaved alike in agglutinin absorption tests. Frobisher and Denny (1928), Elser and Thomas (1936), and Sherman and Stark (1931) found that hemolytic and nonhemolytic strains resembled each other in every respect excepting the reaction on blood. The literature records many instances of the loss of hemo-

TABLE 2  
Characteristics of the enterococci

NO.	AGGLUTINATION IN SERUM			SENSITIVITY TO FILTERED PHAGE		BETA HEMOLYSIS	LIQUEFACTION OF GELATIN	METHYLENE BLUE*	SURVIVAL 60 C, 30 MIN	ACID PRODUCTION FROM						
	1188	1130	894	D/693	D <sub>2</sub> /1188					Arabinose	Sucrose	Lactose	Raffinose	Mannitol	Glycerol	Sorbitol
<i>Streptococcus zymogenes</i> (MacCallum and Hastings)																
1188	500†	100	1,000	+	+	+	-	+	+	-	+	+	-	+	+	+
1308	500	100	1,000	+	+	+	-	+	+	-	+	+	-	+	+	+
914	500	100	1,000	+	+	+	-	+	+	-	+	+	-	+	+	+
1275	500	1,000	1,000	+	+	-	-	+	+	-	+	+	-	+	+	+
1276	500	1,000	1,000	+	+	-	-	+	+	-	+	+	-	+	-	+
1278	500	1,000	1,000	+	+	-	-	+	+	-	+	+	-	+	-	+
1600	100	100	500	+	+	-	+	+	+	-	+	+	-	+	-	+
1355	10	100	10	+	+	+	+	+	+	-	+	+	-	+	+	+
693	100	50	-	+	+	+	-	+	+	-	+	+	-	+	+	+
702	500	100	1,000	+	-	+	-	+	+	+	+	+	+	+	+	+
912	500	1,000	+	+	-	+	-	+	+	-	+	+	-	+	+	+
1357	500	-	1,000	+	-	+	-	+	+	-	+	+	-	+	+	+
1588	100	-	100	+	-	-	+	+	+	-	+	+	-	+	-	+
1359	500	100	-	+	-	+	-	+	+	-	+	+	-	+	+	+
980	100	10	100	+	-	+	+	+	+	-	+	+	-	+	+	+
1531	100	100	500	+	-	-	-	+	+	-	+	+	-	+	+	+
913	100	100	10	+	-	+	+	+	+	-	+	+	-	+	+	+
Group 2																
1130	-	10	100	+	-	+	-	+	+	-	-	+	-	+	+	-
1122	-	10	100	+	-	+	-	+	+	-	-	+	-	+	+	-
1121	10	50	10	+	-	+	-	+	+	-	-	+	-	+	+	-
1131	-	10	-	+	-	+	-	+	+	-	-	+	-	+	+	-
977	10	10	-	+	-	+	+	+	+	+	+	+	-	+	+	-
978	-	1,000	-	-	-	+	-	+	+	-	-	+	-	+	+	+
1187	-	100	-	+	-	-	-	+	+	+	+	+	-	+	+	+
696	-	100	1,000	+	-	+	-	-	+	-	+	-	-	+	+	+
Group 3																
1132	-	-	100	+	-	+	-	+	+	-	-	+	-	+	+	-
979	-	-	100	+	-	+	-	+	+	-	-	+	-	+	+	+
894	-	-	1,000	+	-	+	-	+	+	-	-	+	-	+	+	+
945†	-	-	10	-	-	-	-	+	+	+	+	+	-	+	-	+
Group 4																
1181	-	-	-	+	+	+	-	+	+	-	+	+	-	+	+	+
1574	-	-	-	+	+	-	+	+	+	-	+	+	-	+	+	+
Group 5																
1332	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-	+
1333	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-	+
<i>S. durans</i> (Sherman and Wing)																
1309	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-

\* Strong reduction in milk containing 0.1 per cent methylene blue.

† The titers are expressed as reciprocals of the highest serum dilution which agglutinated.

lytic power in streptococci (Grinnell, 1928; Todd, 1928; Fry, 1933; Lancefield, 1934). That this phenomenon may occur in enterococci was observed by several investigators (Gordon, 1922; Stein, 1933; Noël, 1934). Our strain 1333 was originally a hemolytic strain which, when it was received, had a history of having produced a green variant from a pure line culture. In our laboratory neither strain 1333 nor the substrain 1332 produced beta hemolysis. The reverse change, the acquisition of hemolytic power by enterococci of the alpha type, was reported by Meyer (1926).

In our laboratory repeated changes in type of hemolysis were observed in strain 693. This strain had been isolated by Kendrick and Hollon (1931) from feces in a case of intestinal hemorrhages. They noted that when first isolated it was strongly hemolytic, but that it soon lost its hemolytic power and became alpha hemolytic. After transmission to our laboratory, however, it produced beta hemolysis. Because it did not behave in accordance with its previous history, another subculture of the strain was requested. It also produced beta hemolysis in our laboratory when first received, and it has done so consistently. However, a subculture of our beta hemolytic 693 was given to another laboratory of the National Institute of Health, and it was reported as having changed to an alpha hemolytic strain. A subculture of this alpha strain was returned to our laboratory. It was tested for type of hemolysis on agar containing rabbit blood, which was in general use in our laboratory, and on sheep blood, which was in use in the other laboratory. On rabbit blood agar, beta hemolysis occurred, but on sheep blood, alpha hemolysis occurred. This observation of differences in the type of hemolysis dependent on the source of blood was made also by Kobayashi (1940), who reported that the enterococcus does not hemolyze the blood corpuscles of the goat or sheep, although it may hemolyze the corpuscles of man, horse, cow, or rabbit.

The inconstancy of hemolysis in strain 693 illustrates the unreliability of the hemolytic property as a character of specific significance. The lack of correlation between type of hemolysis and other characteristics is illustrated in table 2 by strains 914 and 1275, which gave almost identical reactions in all tests excepting that for hemolysis.

*Enterococcal bacteriophage.* That enterococcal bacteriophage may be more widespread than phages attacking other streptococci is suggested by the more frequent mention of it in the literature. Beckerich and Hauduroy (1922), and also Hadley and Dabney (1926), studied this phage. Bagger (1926) reported the sudden appearance of bacteriophage in a plate culture of a strain of enterococcus which had been under cultivation for a long time, many similar plate cultures having been made previously. According to Houston (1936), an active enterococcal phage can often be isolated from the stools in cases of ulcerative colitis. This author also reported (1934) that in a septic focus the enterococcus usually occurs in a phage-infected form. He believes that the action of the phage results in variation in the characteristics of the organism. Graham and Bartley (1939) found that 34 of 36 strains of enterococci were sensitive to all three phages which they studied. In our experience, enterococcal phage could be readily obtained from sewage.

Kendrick and Hollon (1931) noted the parallelism between serologic and bacteriophagic relationships in a group of fecal streptococci. One of us (A. C. E.) studied their phage, and reported (1934) that sensitivity to this phage, designated D, differentiated the enterococci from other streptococci, and that on the basis of bacteriophagic reactions the grouping of enterococci corresponded with the grouping recognized on the basis of other characteristics.

During the course of our studies on bacteriophage, a race designated D<sub>2</sub> was found in a sample of sewage taken in Washington, D. C. It lysed enterococci, but differed from phage D in that the antilyns prepared against the two phages behaved differently. Antilysin D<sub>2</sub> neutralized phage D as well as phage D<sub>2</sub>, whereas antilysin D neutralized the homologous phage but not phage D<sub>2</sub>.

Each phage was prepared by propagation on a strain of enterococcus found to be highly sensitive. Phage D was propagated on strain 693, and phage D<sub>2</sub> was propagated on strain 1188. The techniques of isolating the phage, preparing the filtrates and the antilyns, making the serologic tests, and determining the sensitivity of the streptococci to the phages were described in previous publications (Evans, 1934, 1942; Evans and Sockrider, 1942). All strains of enterococci were tested for sensitivity to filtrates of both phages, D and D<sub>2</sub>, with results as shown in table 2.

*Serologic relationships.* A number of investigators (Gordon, 1922; Durand and Dufourt, 1923; Meyer and Löwenstein, 1926; Takeda, 1935; Meyer, 1937) reported that the majority of enterococci from various sources fall into a few well-defined groups according to agglutinative reactions, which were confirmed by agglutinin absorption tests in the studies of some of the investigators.

Saunders (1930) reported that in a large series of cases enterococci from the tissues of resected gastric and intestinal ulcers and from certain types of ulcers in other parts of the body exhibited similar serologic characteristics. Torrey and Montu (1936) found that enterococci serologically related to Saunders' strains occurred more frequently in patients showing intestinal lesions than in normal adults. A few of Saunders' strains (nos. 1275, 1276, and 1277 of tables 1 and 2) and a few of Torrey and Montu's strains (nos. 977, 978, 979, and 980) were available for the present study. It was found that the strains from cases of ulcer, received from Saunders and from Torrey and Montu, resembled strains from other pathologic as well as nonpathologic sources in serologic behavior as well as in physiologic and biochemic reactions.

That the commonest serologic types of enterococci are widely distributed is suggested by the studies of the following authors: Houston (1936) reported that type 1 of the "Belfast classification" was identical with one of Meyer's types of continental European strains, and Meyer (1937) reported that his type 1 agreed with Takeda's (1935) type 1 of strains isolated in Japan.

Agglutinating serums were prepared against three strains, 894, 1130, and 1188, strain 1188 having been selected because it was the strain previously utilized for the propagation of phage D<sub>2</sub>. Strain 1130 was selected to represent strains which failed to agglutinate in antiserum 1188; strain 894 was selected to represent strains which failed to agglutinate in either serum.

inoculating the culture intravenously. Thomson and Thomson (1927) mentioned the isolation of enterococci from the respiratory tract of dogs suffering from distemper. One of the hemolytic strains of our collection, no. 894, was isolated from the diseased lung of a dog, where it was associated with a non-hemolytic streptococcus which the writers did not have an opportunity to study.

*The sources of the enterococci found in human infections.* The data in tables 1 and 2 indicate that strains of enterococci similar to those found in pathologic lesions of man may be derived from the flora of the normal human intestines, or from animal sources. Takeda (1935) also was unable to find differences between the enterococci of the healthy and those of diseased intestines.

Among the 17 strains designated *S. zymogenes*, one was apparently of bovine origin, having been isolated from pasteurized milk; 2 were apparently from nonpathologic human sources, pooled normal plasma; 3 were from normal stools; and the remaining 11 were from human pathologic sources. Among the 8 strains of group 2, one was from a normal throat, one was from an unknown human source, and the remaining 6 were from human pathologic sources. Among the 4 strains of group 3, 2 were from animal sources, and 2 were from human pathologic sources. It may be noted that strain 894, from the diseased lung of a dog, behaved in every reaction like strain 979, from a human case of chronic ulcerative colitis.

No evidence that *S. durans* is pathogenic for man has yet appeared. None of our 23 strains from human pathologic sources showed the characteristics of this species. Brown and Schaub (1945) reported finding one strain of *S. durans* among 386 strains of group D isolated from autopsy material, but they did not state that they found evidence that it had been involved in any disease process.

*The enterococci in human disease.* The literature on enterococci in human disease contains many extensive reviews (Schmitz, 1912; Meyer and Schönfeld, 1926; Thomson and Thomson, 1927; Dible, 1929). The German literature on the pathologic significance of the enterococci was reviewed by Ehrismann (1935). In the following review reference will be made to Ehrismann but not to the authors whom he quotes. This review will omit references to papers in which the description of strains leaves doubt as to whether they were enterococci.

That enterococci are more frequently associated with human disease than they were formerly believed to be is indicated by the report of Brown and Schaub (1945), who stated that nearly 50 per cent of strains of streptococci isolated from autopsy material belonged to group D. These investigators were of the opinion that many of the strains were derived from post-mortem enterococcal invasion, but that many were associated with disease. Rantz and Kirby (1943) reported the finding of many enterococcal infections.

*Enterococci in the human digestive tract.* That the enterococci are common inhabitants of the normal human intestines was demonstrated by Andrewes and Horder (1906) and by many subsequent investigators. Rantz and Kirby (1943) reported that they found enterococci to be constantly present in the normal bowel. On the other hand, Schmitz (1912) failed to find them in normal stools.

Thiercelin (1899) found enterococci involved in enteritis and concluded that they are important in most affections of the digestive tract. Sherman, Stark, and Mauer (1937) investigated the occurrence of *S. zymogenes* in a subject who harbored the organism. It could not always be isolated from the stools, but it could be isolated frequently, and usually with ease, during periods of intestinal disturbance (strain 1188 of tables 1 and 2 was from this case). Ross and Peckham (1920) reported finding enterococci in the stools during an outbreak of 12 cases of severe dysentery, 5 of which were fatal.

Linden, Turner, and Thom (1926) were the first to report outbreaks of food poisoning due to streptococci in cheese. Others have been reported more recently. The streptococcus from one of the early outbreaks was identified with Lancefield's group D by Sherman, Smiley, and Niven (1943). According to Sherman (1945), symptoms similar to those in man may be produced in cats by feeding them with milk in which enterococci have grown.

Saunders (1930) reviewed the literature on the association of streptococci with ulcers of the digestive tract, and he reported finding the constant occurrence of enterococci in the tissues of resected gastric, duodenal, and gastrojejunal ulcers. His observations were confirmed by Torrey and Montu (1936).

According to Felsen (1936), after an acute infection with "*Bacterium dysenteriae*" a secondary infection with enterococci and "*B. coli*" sometimes takes place at the site of the mucosal ulcerations, presumably originally produced by the toxin of the dysentery organism.

Strain 693 of our collection (see the tables) was isolated from feces in a case of intestinal hemorrhage.

*Purulent abdominal infections with enterococci.* Thiercelin's findings (1899) led him to believe that enterococci are important in the production of appendicitis. A number of more recent investigators have confirmed this association. (Meyer and Löwenstein, 1926; Ehrismann, 1935; Elser and Thomas, 1936; Lodenkämper, 1937; Muroi, 1938).

Enterococci are sometimes associated with purulent infections resulting from a damaged intestine or bladder (Schmitz, 1912; Rantz and Kirby, 1943; Wheeler and Foley, 1943; Brown and Schaub, 1945). Strain 1130 of our collection was from a case of peritonitis.

*The streptococci of wounds.* Fleming (1915) was of the opinion that the streptococci as well as other bacteria of wounds inflicted during war are generally of intestinal origin. Burnet and Weissenbach (1918) reported finding enterococci in 30 per cent of osteomuscular war wounds of less than 7 days' duration. Dible (1921) quotes a number of authors who noted the presence of enterococci in wounds studied during the First World War, and other more recent investigators have reported enterococcal infection of wounds (Morin, Caudière, and Certonciny, 1924; Takeya, 1938; Francis, 1941).

*Infections of the urinary tract.* Enterococcal infections of the urinary tract were reported by Andrewes and Horder (1906), Meyer and Löwenstein (1926), Ehrismann (1935), and Elser and Thomas (1936). That enterococci occur frequently in genitourinary infections was shown by Porch (1941), who found

that 73 out of 100 strains of streptococci isolated from specimens of urine belonged to group D. Hollander (1942) found that 22 out of 40 (55 per cent) of streptococci from infections of the genitourinary tract belonged to group D. Rantz and Kirby (1943) found 27 per cent of streptococci isolated from specimens of urine belonged to group D. From 5 of their cases which presented signs of pyelonephritis they isolated enterococci in pure culture.

*Puerperal sepsis.* A good many authors have reported finding enterococci associated with puerperal sepsis (Meyer and Löwenstein, 1936; Ehrismann, 1935). Gordon (1922) found them in 8 cases, Ramsay and Gillespie (1941) in 6, and Rantz and Kirby (1943) in 2. Witebsky and his coworkers (1939) isolated a strain of enterococcus from the blood in a case of septicemia following abortion.

Takezawa (1937) found 50 strains of enterococci among 216 strains of streptococci from female genital organs in various diseases. Hare and Colebrook (1934) isolated streptococci with the characteristics of enterococci from 7 of 34 women who had low-grade fever during the puerperium, but in only a few instances were the organisms isolated in pure culture. Lancefield and Hare (1935) found no streptococci of group D among 46 strains from cases of severe infection of the uterus, but they found 8 strains of group D among 18 strains from "minor infections." Brown and Schaub (1945) found that 9 per cent of 232 strains of streptococci from the uteri of patients with febrile puerpera were enterococci.

*Otitis media, mastoiditis, and meningitis.* Thiercelin (1899) isolated enterococci from cases of meningitis, and Andrewes and Horder (1906) found them in cases of otitis media, mastoiditis, and meningitis. Subsequent investigators have confirmed those early reports. Ehrismann (1935) quoted two authors who reported cases of meningitis due to enterococci; Lang, Lode, and Schuttermayer (1937) reported 2 cases; Wheeler and Foley (1943), 1 case. Rantz (1942) reported 1 case in which meningitis followed a prolonged ear infection. Rantz and Kirby (1943) found that about 10 per cent of streptococcal infections of the middle ear were caused by enterococci.

Among the strains of our collection, no. 1132 was from a case of ear infection following measles; no. 1181 was from a case of meningitis following mastoiditis; and no. 1574 was from a case of otitis media.

*Endocarditis.* MacCallum and Hastings (1899) obtained an organism which they called *Micrococcus zymogenes* from the blood in a case of endocarditis, and Sherman (1937) was convinced that their organism was an enterococcus. Subsequently many authors reported the isolation of enterococci in cases of endocarditis (Andrewes and Horder, 1906; Hicks, 1912; Gordon, 1922; Meyer and Löwenstein, 1926; Dible, 1929; Wallach, 1934; Houston, 1934; Baum, 1935; Ehrismann, 1935; Elser and Thomas, 1936; Reiners, 1936; Fox, 1936; Waaler, 1937; Clements, 1937; Otto, 1938; Moran, 1938; Rohleder, 1938; Williams, 1939; Lederle, 1940; Skinner and Edwards, 1942; Rantz and Kirby, 1943; Wheeler and Foley, 1943; MacNeal and Blevins, 1945; Brown and Schaub, 1945). Two strains of our collection, nos. 1332 and 1333, were from one case of malignant endocarditis.



Some of the investigators mentioned reported on the frequency of occurrence of enterococci in their cases of endocarditis. Elser and Thomas encountered enterococci "not infrequently" in the blood of patients suffering from a subacute form of endocarditis. Andrewes and Horder found 4 strains of enterococci among the streptococci from 24 cases of malignant endocarditis; Dible reported that 1 strain out of 6 isolated from the blood in cases of ulcerative endocarditis was an enterococcus; Moran found it in 5 out of 20 cases; Lederle in 8 out of 10 cases; Rantz and Kirby in 3 out of 16 cases; and MacNeal and Blevins in 6 out of 36 cases.

The portal of entry was determined in a number of cases of endocarditis reviewed by Skinner and Edwards (1942). Enterococci obtained from the blood stream were derived from an infected finger in 1 case, infected tonsils in 1, the gall bladder in 2, the urinary tract in 2, septic abortion in 3, and the gastrointestinal tract in 5.

*Miscellaneous diseases.* Rantz and Kirby (1943) called attention to the fact that the enterococci are rarely found associated with infections of the respiratory tract. They reported 8 cases; Wheeler and Foley (1943) reported 1 case. Brown and Schaub (1945) found enterococci in mixed cultures in cases of pneumonia. One strain of our collection (no. 702) was from a case of sore throat.

The enterococcus is occasionally reported to be associated with various diseases not mentioned above. Meyer and Löwenstein (1926) found it in cholecystitis, osteomyelitis, and pancreatitis; Houston (1934) found it in septic tonsils, the root canal of septic teeth, postnasal catarrh, septic antra, excised gall bladders, abscesses in various parts of the body, certain forms of acne and other skin lesions, and invariably in chronic onychia; Wheeler and Foley (1943) found it in dermatomyositis and in emphysema. Our collection includes strains from empyema (no. 696), an infected tooth (no. 912), osteomyelitis (no. 1121), and septicemia following smallpox vaccination (no. 1131).

There is an extensive literature on the association of enterococci with rheumatic diseases. It is omitted here because, if the association should be proved to be significant, this literature should be treated separately.

#### SUMMARY

The literature on human infections with enterococci is reviewed, and the results of a study of 34 strains, 23 from human pathologic sources and 11 from other sources, are reported.

Enterococci have been found in a great variety of human ailments. They appear to be important causal agents in some cases of endocarditis, intestinal disorders, abdominal infections due to injury of the intestinal tract, infections of wounds inflicted during war, and infections of the urinary tract.

The following characteristics distinguish the enterococci from other streptococci: reaction in serum of group D according to Lancefield's precipitin test, growth at 10 and 45 C, growth in media containing 6.5 per cent sodium chloride, growth in media having an initial pH value of 9.6, and growth in media containing 40 per cent bile.

The hardness of the enterococci is of practical significance in that they are resistant to the sulfonamides and penicillin.

The following characteristics are useful in distinguishing subgroups or species of enterococci: agglutinative reactions; sensitivity to bacteriophage D-693; growth in milk containing 0.1 per cent methylene blue; survival at 60 C for 30 minutes; and the production of acid from sucrose, mannitol, and sorbitol.

The type of hemolysis and liquefaction of gelatin, characteristics on which the differentiation of species hitherto have been based, were found to be uncorrelated with other significant characteristics.

The data indicate that enterococci pathogenic for man may be derived from the flora of normal human intestines or from the tissues or intestines of various species of animals.

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# ANTIBIOTIC ACTIVITY OF THE FATTY-ACID-LIKE CONSTITUENTS OF WHEAT BRAN

HARRY HUMFELD

*Western Regional Research Laboratory,<sup>1</sup> Albany, California*

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An appreciable part of the antibiotic activity observed in connection with the culturing of an unidentified microorganism on a medium consisting principally of wheat bran and asparagus juice was found to reside in the medium itself. Investigation disclosed that the wheat bran was the source of this activity. Wheat bran has frequently been used as an ingredient in media assayed for antibiotic activity. Because of the possibility of confusing the antibiotic activity naturally present in wheat bran with the activity produced by microorganisms grown on media containing wheat bran, efforts were made to characterize the active factor involved. A hypothesis is presented that antibiotic activity may be formed by the hydrolysis and saponification (by the action of the microorganisms) of fatty constituents contained in the original substrates.

## EXPERIMENTAL PROCEDURE

Preliminary observations indicated that the constituent or constituents of wheat bran possessing antibiotic activity were extractable with 60 or 95 per cent ethanol, petroleum ether, or diethyl ether, but were not appreciably water-soluble. These extracts, particularly those obtained by the use of petroleum ether or diethyl ether, when saponified with KOH, yielded soaps which had even greater activity on an equivalent basis than the original bran. Extraction of the hydrolyzed material with diethyl ether to obtain the neutral and the acid ether-soluble fractions showed that all of the active material was present in the latter.

A quantity of the antibiotically active fraction was obtained by extracting 1,000 grams of wheat bran overnight in a percolator with 2.5 liters of petroleum ether, following which procedure the fraction was drained and washed with an additional 1.5 liters of the ether. These extracts were combined and evaporated almost to dryness on a steam bath. The residue was extracted with aliquots of 95 per cent ethanol, totaling about 300 ml. Three hundred ml of 0.832 *N* alcoholic KOH were added to the ethanol solution, and the mixture was refluxed for 2 hours. The refluxed solution was concentrated to about 200 ml and then diluted with 1,600 ml distilled water. This solution was then extracted with five 200-ml portions of diethyl ether to remove the "neutral" ether-soluble fraction. The alcohol-water solution was then acidified with HCl to pH 2, and the diethyl ether extraction was repeated. After being washed with water, the combined acid-ether extracts were evaporated, from which about 30 g of a brown, oily residue were obtained. This residue, which contained the active material,

<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

was then extracted with 95 per cent ethanol. The ethanol-soluble fraction was removed by centrifuging the suspension of the residue in the ethanol, and a potassium salt was prepared from the supernatant by the addition of 90 ml of 0.096 N KOH. This solution, after dilution with about 600 ml of water, was shell-frozen in round-bottomed boiling flasks and then dried under vacuum from the frozen state. The final yield was 27 g.

Tests for antibiotic activity were made on each extract at each step of the separation, and a control containing only the solvent was made on each solvent. It was calculated that the final product contained about 95 per cent of the original activity. It was found that the refluxed alkaline alcohol solution was more active than the original petroleum ether extract, which was probably due to the hydrolysis of some of the fats to free fatty acids. The solvent controls showed no activity at the inhibition levels of the active extracts.

A modified medium II of Schmidt and Moyer (1944) was used for the bioassay. The ingredients of the medium were peptone, 5 g; yeast extract, 1.5 g; beef extract, 1.5 g; "N-Z-amine" type B, 2.0 g; glucose, 3.0 g; NaCl, 3.5 g; 500 ml of  $\text{KH}_2\text{PO}_4$  (15 g per liter) adjusted to pH 7.0 with NaOH; and distilled water to make 1 liter. The medium was placed in bottles of convenient size, usually in the amount required for any one series of assays. The inoculum was grown by transferring it from an agar slant to 100 ml of the medium in a 250-ml Erlenmeyer flask, and incubating it 18 hours at 37 C. The inoculum was added to the medium at the rate of 20 ml per liter and the serial dilution set up. Two logarithmic series of 10, 100, 1,000, etc., and 5, 50, 500, etc., were set up for the survey assay during the fractionation. When more accuracy was desired, as in the comparison of solutions of various salts of the fatty acids, series of 120, 200, 300, 400, 600, 800, and 1,200, or 10-fold multiples thereof, were set up. Test tubes 18 by 150 mm in size were used. Each series was made in duplicate and inoculated, and sterile control blanks were set up with each series. The tubes were incubated for 4 hours in a water bath at 37 C. At the end of the incubation period the tubes were sterilized, and the turbidity of each tube was measured in a Klett-Summerson colorimeter. The turbidity readings were plotted against the dilutions on a semilogarithmic paper. A sigmoidal inhibition curve was obtained. The point of 50 per cent inhibition as compared with the readings of the antibiotic-free controls was taken as the most accurate measure of degree of inhibition. From the dilution at which this point in the curve occurred the concentration in micrograms per milliliter was calculated. For the comparison of the activity of the wheat bran fraction with some other salts of fatty acids, *Staphylococcus aureus* (Food and Drug Administration strain 209), *Micrococcus conglomeratus* (Merck's N.Y. strain), *Streptococcus faecalis* (ATCC 7080), and *Escherichia coli* (Waksman's strain for testing streptomycin) were used as the assay organisms.

Potassium laurate, sodium oleate, potassium salt of mixed acids of castor oil, potassium salt of the mixed acids of cottonseed oil,<sup>2</sup> and a sample of potassium

<sup>2</sup> Samples of these were supplied by Dr. Ernest Kester of this laboratory.



linoleate made from methyl linoleate<sup>3</sup> were used for the purpose of comparing activities.

### RESULTS

Extracts obtained by treating separate 10-g samples of wheat bran with 200-ml portions of water, and with 70 per cent ethanol, produced a 50 per cent inhibition of *S. aureus* at dilutions of 10 and 260, respectively. A comparison with various other salts of fatty acids readily available is given in table 1. The results indicated that the potassium salt obtained from the wheat bran was considerably more active than any of the other salts tested, with the exception of potassium linoleate, which had about the same activity as the salts of the wheat bran extracts.

TABLE 1  
*Antibiotic activity of the salts of fatty acids from various sources*

FATTY ACID SALTS	CONCENTRATION OF SALTS GIVING 50 PER CENT INHIBITION OF		
	<i>S. aureus</i>	<i>M. conglomeratus</i>	<i>S. faecalis</i>
	μg per ml	μg per ml	μg per ml
Potassium laurate.....	22	16	27
Sodium oleate.....	23	18	100
Potassium salts of mixed acids of castor oil*.....	50	38	45
Potassium salts of mixed acids of cotton seed oil*..	50	41	48
Potassium salt of acid ether fraction of wheat bran..	4	5.5	10
Potassium linoleate.....	3.5	4.2	6

\* These samples were stored laboratory samples, and it is likely that freshly made samples would have shown higher activity.

Results with *E. coli* indicated no inhibition within the range tested. In fact, with the wheat bran salt, a definite stimulation was noted at 300 micrograms per milliliter. These findings would seem to indicate that these salts would probably be more active against gram-positive organisms than against gram-negative ones.

### DISCUSSION

Germicidal and bacteriostatic activity of some fatty acids is well known. Stimulatory action of these and similar materials at certain concentrations also has been reported. Whether the action will be stimulatory, inhibitory, or germicidal apparently depends upon the kind and concentrations of the materials added, on the physical and chemical environment, and on the type of organism employed. In riboflavin assays, an alcoholic extract of fresh liver hydrolyzed with alkali was found to be strongly inhibitory for *Lactobacillus casei* (Feeney and Strong, 1942). Feeney and Strong also found that ether extract of whole blood was stimulatory at low, and inhibitory at higher, concentrations. Kodicek

<sup>3</sup> Supplied by Dr. Gordon Rose of the Enzyme and Phytochemical Research Division of this bureau.

and Worden (1944), also studying factors affecting the riboflavin assay, found that *Lactobacillus helveticus* was inhibited for 24 hours by oleic acid, and for 72 hours or more by linoleic and linolenic acids, when used in concentrations of 160 micrograms per 10 ml of culture, or at 16 parts per million. Avery (1918) was able to suppress the growth of pneumococci and streptococci, while attempting to isolate "*B. influenza*," by adding sodium oleate to a hemoglobin medium. The activity of various fatty acid soaps was tested by Lamar (1911a, 1911b, 1912). Lysis of pneumococci was obtained at comparatively high dilutions of sodium oleate, potassium linoleate, and potassium linolenate. The latter two salts inhibited growth for 1 hour at dilutions up to 1:4,000 and 1:6,000. It was concluded that the action was directly proportional to the degree of unsaturation of the acid.

Bergström, Theorell, and Davide (1946) found that the presence of fatty acids in the medium interfered with the oxygen uptake of *Mycobacterium tuberculosis*. Di-heptylacetic acid reduced oxygen uptake at a 1:7,000 dilution, whereas the uptake was completely inhibited by oleic acid at 1:10,000, by linoleic acid at 1:15,000, and by linolenic acid at 1:30,000. After extended investigation, Stanley, Coleman, Greer, Sacks, and Adams (1932) concluded that the most active compounds were aliphatic acids which contain from 15 to 18 carbon atoms. They studied the action of chaulmoogra oil and related compounds on *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and other acid-fast bacteria. The most effective acids were good surface tension depressants. This physical property seemed to be more important than the detailed chemical structure. The sodium salts of these acids were found to be effective in dilutions of 1:50,000, or at 20 parts per million.

Barton-Wright (1938) reported that, in the fatty fraction of wheat bran, the total combined acids are 84 per cent unsaturated, with an iodine value of 152.4. It seems safe to assume the presence of a considerable amount of linoleic acid.

The separation from wheat bran of a material with antibiotic properties, which is of a fatty acid nature or is closely associated with the fatty acid, has some rather interesting implications. For instance, when wheat bran, which contains fatty acid constituents, is extracted with 70 per cent ethanol, antibiotic activity is obtained in the extract. Also, the possibility no doubt exists that such fatty constituents may be hydrolyzed by the action of the microorganisms and subsequently saponified. When the culture is subsequently assayed, water-soluble antibiotic activity may be found, and such activity may be attributed to the antibiotic normally produced by the microorganism.

Keeping this hypothesis in mind, it might be well to re-examine the findings reported by Srinivasa (1944), Mohan *et al.* (1946), Moyer and Coghill (1947), and Holtman (1945). For instance, extracts obtained by the extraction of Moyer and Coghill's (1947) wheat bran medium with 70 per cent alcohol at pH 7.5 gave a 50 per cent inhibition of *S. aureus* at 1:200, of *S. faecalis* at 1:108, and of *M. conglomeratus* at 1:60. Hence, if an organism capable of hydrolysis of the fatty constituents of the medium were grown, a water-soluble salt having antibiotic activity might be formed, which, when the medium was assayed, could give a false picture of antibiotic activity produced by the organism. Actual separation

of this fraction from the antibiotic produced by the organism would then be necessary in order to obtain the correct data. Intensive search of the literature might bring to light numbers of instances in which the addition to media of fat- and fatty-acid-containing materials resulted in an increase in antibiotic activity.

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#### SUMMARY AND CONCLUSIONS

A fraction having antibiotic properties was extracted from wheat bran. This material has the characteristics of a fatty acid and forms a water-soluble potassium salt which has a comparatively high activity against *Staphylococcus aureus*, *Micrococcus conglomeratus*, and *Streptococcus faecalis*. It was inactive against *Escherichia coli*. When materials of plant or animal origin containing fats or fatty acid constituents are used in making microbiological media, the possibility of these constituents having antibiotic activity, which might be confused with activity produced by microorganisms, should be given consideration.

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# RIBOFLAVIN PRODUCTION BY MOLDS<sup>1</sup>

GEORGE L. PELTIER AND RAYMOND BORCHERS

*Departments of Bacteriology and Agricultural Chemistry, University of Nebraska, Lincoln, Nebraska*

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Very little information is at hand regarding the ability of molds to synthesize riboflavin. Few citations pertaining directly to riboflavin production by true molds are available.<sup>2</sup> Pontovich (1943) found as much as 2 mg riboflavin per g of *Aspergillus flavus* mycelium. Tanner *et al.* (1945) determined the quantity of riboflavin in the submerged fermentation media of *Penicillium chrysogenum*. The highest value found was 1.36 mg per ml. The primary purpose of this study was to screen several hundred isolates, recently obtained from soil, crop residues, and composts, for their ability to produce riboflavin on a wheat bran substrate.

## METHODS

*Preparation and inoculation of wheat bran.* Ten grams of wheat bran and 10 ml of water were thoroughly mixed in 12-oz French squares. The bottles were then plugged with cotton and autoclaved for 60 minutes at 121 C.

The mold isolates were carried on potato glucose slants, and the inoculum was prepared as follows: Several grams of sterile, moistened wheat bran (in test tubes) were inoculated directly from the agar slants and allowed to sporulate well. Approximately 0.5 g of this dried mold bran served as the inoculum for each bottle. The bottles were incubated horizontally at 30 C until good mycelial growth was obtained (72 to 96 hours).

*Riboflavin assay.* The dry mold bran was assayed for riboflavin by the *Lactobacillus casei* acid production method of Snell and Strong (1939) as modified by Strong and Carpenter (1942).

## RESULTS

The results of the screening tests are presented in table 1. Of the 240 isolates, all were capable of riboflavin synthesis. As will be noted, however, some genera are better able to produce riboflavin than others. The isolates of the genus *Fusarium* are rather outstanding in this respect, as well as are certain of the aspergilli. The most outstanding isolate was a "gold" *Aspergillus* which yielded a value of 5.8 mg riboflavin per 100 g of mold bran.

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<sup>2</sup> The commercial applications of the so-called *Eremothecium ashbyii* and *Ashbya gossypii* in riboflavin production and the patent literature pertaining thereto are not considered in this discussion.

TABLE 1  
*Riboflavin production by molds*

GENUS	NUMBER OF ISOLATES	RIBOFLAVIN PER 100 G MOLD BRAN*			
		0.40-0.49 mg	0.50-0.99 mg	1.00-1.99 mg	2.00+ mg
<i>Aspergillus</i>					
black.....	47	0	2	39	6
green.....	19	0	2	17	0
tan.....	27	0	10	8	9
gold.....	16	0	2	9	5
misc.....	4	0	1	2	1
<i>Penicillium</i>					
blue-green.....	27	0	11	14	2
gray-green.....	19	0	10	7	2
yellow-green.....	11	2	5	4	0
compact raised.....	15	2	5	7	1
<i>Alternaria</i> .....	4	0	2	2	0
<i>Fusarium</i> .....	26	0	3	7	16
<i>Hormodendrum</i> .....	9	1	5	3	0
<i>Rhizopus-Mucors</i> .....	11	0	6	4	1
<i>Trichoderma</i> .....	5	0	0	3	2
Total.....	240	5	64	126	45

\* From 0.25 to 0.35 mg riboflavin per 100 g wheat bran before molding.

#### SUMMARY

Of the 240 fungal isolates grown on a wheat bran substrate, all were capable of producing some riboflavin. Forty-five isolates gave values in excess of 2 mg per 100 g of mold bran. Certain isolates of the genera *Fusarium* and *Aspergillus* were particularly outstanding. The highest yield of riboflavin, 5.8 mg per 100 g of mold bran, was obtained from a "gold" *Aspergillus*. It can be concluded that riboflavin synthesis is rather common, at least in the molds studied, and that certain isolates produce riboflavin in amounts sufficient to warrant further study as a biological source of riboflavin.

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# STUDIES ON THE MODE OF ACTION OF STREPTOMYCIN

## II. THE NATURE OF A STREPTOMYCIN INHIBITOR OCCURRING IN BRAIN TISSUE AND PLANT EXTRACTS<sup>1</sup>

IONE RHYMER AND G. I. WALLACE

*Department of Bacteriology, University of Illinois, Urbana, Illinois*

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The action of streptomycin on *Eberthella typhosa* and *Staphylococcus aureus* was found by Wallace, Rhymer, Gibson, and Shattuck (1945) to be much greater in a poor medium (nutrient broth) than in a good medium (brain heart infusion). An interpretation was made that "there is something present in the brain heart infusion which interferes in some way with the action of streptomycin which is not present in nutrient broth." Similar observations have been reported by others and in some cases different interpretations have been made. Berkman, Henry, and Housewright (1947), for example, believe that the resistant individuals in any one test grow out faster in the better medium than they do in the poorer medium and that an interfering substance is not necessarily present. Further studies have been made to determine, if possible, the nature of the interfering substances.

The streptomycin was prepared at the University of Illinois in the laboratory of H. W. Anderson and purified and standardized in the laboratory of H. E. Carter (Carter *et al.*, 1945; Loo *et al.*, 1945). The unit used is equivalent to the FDA unit, which is equal to one microgram of the free base or 0.84 micrograms of the hydrochloride. The crystalline streptomycin was dissolved in the liquid medium under study, thereby eliminating the error that might arise from dilution of the test medium.

*Eberthella typhosa* (Hopkins strain) and *Staphylococcus aureus* (FDA 209) were again used as the test organisms. A 24-hour broth culture of the organism was diluted with twice its volume of sterile water for the inoculum, 0.1 ml of this suspension being used in all cases. In the tests, plate counts were made at the start and 3, 6, 9, and 24 hours later to determine the numbers of viable organisms present.

Because considerable amounts of brain heart infusion were to be used in the studies, the use of Difco brain heart infusion seemed desirable for most consistent results. The brain heart infusion used up to this time was prepared in the laboratory by the authors and did not have any inorganic salts added to it. Since the Difco medium contained both sodium chloride and disodium phosphate, a series of tests were run to see whether the salts in the Difco medium would have any effect upon the streptomycin. The two media gave almost identical results in these tests, so it was concluded that sodium chloride and disodium phosphate in

<sup>1</sup> Summary of a thesis presented in partial fulfillment of the requirements for the Ph.D. degree by the senior author.

the amounts present in Difco brain heart infusion would not interfere with the action of streptomycin.

Since the first published report considered only amounts of streptomycin which would decrease the numbers of microorganisms, this work was continued to determine the concentration of streptomycin necessary to destroy all organisms. It was found that approximately 4.1 units of streptomycin per ml of culture medium would destroy all the added cells of *Eberthella typhosa* in nutrient broth, and that approximately 1 unit of streptomycin per ml of culture medium would destroy all the added cells of *Staphylococcus aureus* in nutrient broth. In brain heart infusion the results were entirely different. A concentration of streptomycin of 50 units per ml of medium failed to destroy all the cells of *Eberthella typhosa* and prevent growth of the organism in the medium. Twenty-five units per ml did not destroy the cells of *Staphylococcus aureus* in the brain heart infusion. In this particular study the plate counts were used to follow the killing action of streptomycin. The results again indicate that a protective substance for the organism under test or an antagonistic substance to streptomycin is present in the brain heart infusion.

In order to determine the nature of this substance Wolf's (1945) "casamino acid" medium was used as a base medium for further study. This medium is constant in composition and is reproducible. The complete dehydrated medium was obtained from Difco Laboratories and also vitamin-free casamino acids for the preparation of the complete medium.<sup>2</sup> In this medium it was found that 40 units of streptomycin per ml of culture and 2.6 units of streptomycin per ml of culture would destroy all the added cells of *Eberthella typhosa* and *Staphylococcus aureus*, respectively. The amount of streptomycin necessary here is considerably more than that needed for destruction in the nutrient broth, but very much less than that needed in the brain heart infusion. This may be interpreted as indicating that the interfering substance or substances are present in the casamino acid medium but not in the same concentration as they are in brain heart infusion. It seemed desirable first to determine the effect of the constituents in the complete casamino acids medium. Uracil, thiamine hydrochloride, and niacinamide are present in the medium, so the complete casamino acid medium was compared with casamino acid medium minus thiamine hydrochloride, casamino acid medium minus niacinamide, casamino acid medium minus uracil, and casamino acid medium minus thiamine hydrochloride, niacinamide, and uracil. The results were all similar, indicating that the constituents present except the casamino acids were not affecting, in any way, the action of the streptomycin. Some inorganic salts are present in the casamino acids medium, but since an earlier study had indicated the unimportance of sodium chloride and disodium phosphate it was thought advisable to postpone study of the salt content and to examine the casamino acids or the nitrogen-containing constituents of culture media for the interfering factor.

At this point it was decided to change the test procedure. Because a good

<sup>2</sup> The authors wish to express their sincere appreciation to Difco Laboratories for the many materials and courtesies given to them.



many substances were to be tested and the plate counting procedure was tedious and time-consuming, it was thought to be more advantageous to change from a measurement of numbers of bacteria present by plate counting to one of presence or absence of turbidity. The culture tubes were examined for presence or absence of turbidity as compared with an untreated culture in the test medium. Some checks were made comparing this type of reading results with plate counts and it was found that sufficiently accurate readings could be made.

*Effect of adding commercial peptones to base medium.* The available commercial bacteriological peptones were then added to the casamino acid medium

TABLE 1

*Action of streptomycin on Eberthella typhosa in casamino acid medium with addition of different peptones*

PEPTONES	CONCENTRATION OF STREPTOMYCIN IN UNITS PER ML																	
	0	3.3	6.7	10.0	13.3	16.7	20.0	21.7	23.3	25.0	26.7	28.3	30.0	31.7	33.3	35	36.7	38.3
0.5% Liver fraction L (W).....	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
1.0% Phytone (B.B.L.)..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
1.0% Neopeptone (D)...	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
1.0% Peptone (D).....	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
1.0% Proteose peptone III (D).....	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-
1.0% Trypticase (B.B.L.).....	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-
1.0% Proteose peptone (D).....	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
1.0% Tryptose (D).....	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
1.0% Tryptone (D).....	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
1.0% Casamino acids (D).....	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	-
Casamino acid medium (D).....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(W) = Wilson Laboratories. (B.B.L.) = Baltimore Biological Laboratory. (D) = Difco Laboratories.

+ = turbidity. - = no turbidity.

and the effect on streptomycin noted. With *Eberthella typhosa* phytone (B.B.L.), liver fraction L (Wilson), trypticase (B.B.L.), and tryptone (Difco) had an inhibitory effect on the action of streptomycin, with the degree of activity in the order listed. Tryptone (Difco), proteose peptone no. 3 (Difco), peptone (Difco), proteose peptone (Difco), and neopeptone (Difco) all inhibited streptomycin slightly. Phytone, liver fraction L, and neopeptone gave the greatest inhibitory action with *Staphylococcus aureus*, whereas the remainder of the peptones had only slight effect. Tables 1 and 2 give these results in detail.

The results at this stage of the investigation all pointed toward the presence of some factor in the nitrogen-containing constituents of culture media which interfered with the action of streptomycin. It was present in varying amounts

but was greatest in brain heart infusion medium. Of the bacteriological peptones, it was present in greatest amount in phytone, a peptone made from plant proteins. Because the brain heart infusion had so much activity, an intensive study was then made of it in an attempt to learn more about the interfering substance.

*Adsorption of brain heart infusion with activated carbon.* Brain heart infusion was treated with "darco" once and tested for action. It was found to contain all of its activity. It was then treated with darco five times and tested, again showing no decrease in its ability to inhibit the action of streptomycin. It

TABLE 2

*Action of streptomycin on Staphylococcus aureus in casamino acid medium with the addition of different peptones*

PEPTONES	CONCENTRATION OF STREPTOMYCIN IN UNITS PER ML																		
	0	1.7	3.3	5.0	6.7	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	
0.5% Liver fraction L (W).....	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	
1.0% Phytone (B.B.L.)..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
1.0% Neopeptone (D)...	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	
1.0% Peptone (D).....	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Proteose peptone III (D).....	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Trypticase (B.B.L.).....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Proteose peptone (D).....	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Tryptose (D).....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Tryptone (D).....	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
1.0% Casamino acid (D).	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Casamino acid medium (D).....	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

(W) = Wilson Laboratories. (B.B.L.) = Baltimore Biological Laboratory. (D) = Difco Laboratories.

+ = turbidity. - = no turbidity.

seems rather definite, therefore, that no vitamin or any substance which would adsorb to the carbon could account for this action.

*Hydrolysis of brain heart infusion.* After hydrolysis with trypsin the brain heart infusion would not support growth of *Eberthella typhosa*, so activity could not be measured directly. *Staphylococcus aureus* did grow on the hydrolyzed brain heart infusion, but not well. Casamino acids, uracil, thiamine hydrochloride, and niacinamide were added to the hydrolyzate in the amounts that are present in casamino acid medium, and growth of both *Eberthella typhosa* and *Staphylococcus aureus* was obtained. When only thiamine hydrochloride and niacinamide were added, the same results were obtained as with the hydrolyzate alone. Liver fraction L was added to the hydrolyzate and the resulting medium gave good growth. Phytone was hydrolyzed and gave results very similar to

those obtained with the hydrolyzed brain heart infusion. The activity of the hydrolyzates in interfering with streptomycin action closely paralleled the growth activity. Apparently the growth factor and the streptomycin-interfering factor are both destroyed by this process, or perhaps the two activities are caused by one substance which is destroyed.

*Study of brain and heart infusions separately.* Difco Laboratories prepare a heart infusion broth culture medium containing infusion from beef heart, tryptose, and sodium chloride. This medium was tested for its streptomycin-inhibiting activity and was found to have none, which indicates very strongly that the activity is present in the brain infusion. Consequently a brain infusion was obtained from two sources, the Difco Laboratories and H. E. Carter, University of Illinois, and both were studied for their activity. The two infusions gave parallel results, indicating, as was suspected, that the activity was present in rather large amounts in brain tissue.

TABLE 3

EXTRACTION	BRAIN HEART INF.		PHYTONE	
	<i>E. typhosa</i>	<i>S. aureus</i>	<i>E. typhosa</i>	<i>S. aureus</i>
Methanol extract.....	++	++	+	+
Residue of methanol extract.....	+	+	++	++
Ether precipitate of methanol extract.....	++	++		
Ether-soluble portion of methanol extract.....	+	+		

+ = presence of antistreptomycin activity.

*Extraction of brain heart infusion and phytone.* Brain heart infusion and phytone were then extracted with methanol and the extract and residue were both tested for activity. When an equal volume of ether was added to the methanol extract of brain heart infusion a precipitate was formed, so the precipitate and the ether-soluble part were tested. Table 3 gives the results of these studies. The results were quite sharp and indicate that in the brain heart infusion, although the extraction was not complete, most of the activity was present in the methanol extract. The ether precipitate also carried most of the activity from the methanol extract. In the phytone the activity was greatest in the residue of the methanol extract, which indicates that the substances in brain heart infusion and phytone are not identical or that something interferes with their extraction by methanol.

*Hydrolysis of methanol extract.* The methanol extract of brain heart infusion was hydrolyzed for 1 and for 4 days and tested for activity. It was found that some activity was destroyed in 1 day and that all activity was destroyed in 4 days.

*Dialysis of brain heart infusion.* Brain heart infusion was dialyzed in a collodion membrane, and the active substance completely dialyzed through the membrane in running water within 3 hours.

The latter tests required an assay procedure which was developed as follows: The substance to be assayed was dissolved in water so that 1 ml contained 12.5 mg of the substance. This solution was added to 4 test tubes in 25-mg, 12.5-mg, 6.25-mg, and 3.125-mg amounts. Each of the tubes was then made up to the 2-ml volume with distilled water. To each were then added 2.5 ml of double strength casamino acid medium, and the whole was sterilized. One hundred and twenty-five units of streptomycin contained in 0.5 ml of water and one drop of a 24-hour broth culture of *Eberithella typhosa* were added to each of the 4 tubes. When *Staphylococcus aureus* was used, each 0.5 ml of the antibiotic solution contained 31.25 units. All tubes were incubated for 15 hours at 37 C and the presence or absence of turbidity was observed. Brain heart infusion cultures were assayed at the same time as a control for comparison.

#### DISCUSSION AND CONCLUSIONS

Antibacterial activity of streptomycin has been shown to be greatly influenced by the composition of the medium in which it is acting. A brain heart infusion shows great ability to inhibit the action of streptomycin. Certain peptones, especially phytone, a peptone made from plant proteins, also have this ability but to a lesser degree. Studies have indicated that the activity is due to something which can be extracted from the media, thus indicating that a specific substance or group of substances is responsible for the inhibitory action. Tests have shown that brain tissue contains large amounts of an active substance. Greater activity was observed with the extract of brain than with the phytone. Further studies are being made to determine its nature.

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## THE ACTION OF PHENYLMERCURIC NITRATE

### IV. THE ABILITY OF SULFHYDRYL COMPOUNDS TO PROTECT AGAINST THE GERMICIDAL ACTION OF BASIC PHENYLMERCURIC NITRATE

GIRARD W. THOMAS AND ELTON S. COOK

*Laboratories of the Institutum Divi Thomae, Cincinnati, Ohio*

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In earlier papers it was shown that yeast extracts have the ability to antagonize the inhibitory action of basic phenylmercuric nitrate on the growth of yeast and bacteria (Cook and Kreke, 1943; Thomas, Fardon, Baker, and Cook, 1945), on the respiration of yeast (Cook and Kreke, 1943), on the growth of molds (Cook and Kreke, 1940), and on the respiration (Cook, Kreke, Eilert, and Sawyer, 1942) and growth (Thomas, Fardon, Baker, and Cook, 1945) of skin.

In attempts to elucidate these findings it was demonstrated that this germicide depresses the activity of cytochrome oxidase, succinic, lactic, and glucose dehydrogenases, and catalase (Cook, Kreke, McDevitt, and Bartlett, 1946). Further experimentation showed that the depression of yeast respiration and of cytochrome oxidase activity by phenylmercuric nitrate, as measured by the Warburg respirometer, could be prevented but not reversed by the sulfhydryl compounds cysteine, homocysteine, and glutathione, whereas cystine and methionine, as well as a number of amino acids containing other reactive groups, were ineffective as protecting agents (Cook, Perisutti, and Walsh, 1946; Cook and Perisutti, 1947).

The present paper demonstrates that the inhibition of the growth of *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* by basic phenylmercuric nitrate can be antagonized by the addition of sulfhydryl-containing compounds. Efforts to reverse the inhibition after exposure of *Escherichia coli* to the germicide have been unsuccessful.

#### EXPERIMENTAL PROCEDURES

*Solid medium: Filter disc method.* In preliminary experiments nutrient agar containing cysteine in concentrations ranging from  $1.5 \times 10^{-6}$  M to  $6.0 \times 10^{-4}$  M was prepared. After sterilization and subsequent cooling to 42 C, the medium was poured over 0.1 ml of a 48-hour nutrient broth culture of the test organism in sterile petri dishes. After solidification of the agar, filter paper discs dipped in phenylmercuric nitrate solutions of a concentration range from  $1.5 \times 10^{-6}$  M to  $1.5 \times 10^{-4}$  M were placed on the surface of the agar. The control medium was nutrient agar without cysteine. Zones of inhibition were measured after 18 hours' incubation at 37 C. There was evidence of antagonism as shown in table 1. This method does not allow comparison of effective concentrations of germicide and cysteine since the actual concentration of phenylmercuric nitrate in contact with cysteine in the medium depends upon diffusion and hence is unknown.

In all subsequent experiments the sulfur-containing compounds (cysteine, homocysteine, glutathione, cystine, or methionine) were mixed with the phenylmercuric nitrate, and the mixture was allowed to stand 5 minutes to insure reaction. The filter discs were dipped in the solution and placed on the surface of the agar. In both of these methods the diameter of the filter paper disc was 12.7 mm so that a 13-mm zone of inhibition represents only trace activity and less than this value is represented in the tables as 0 inhibition.

TABLE 1

*Growth of organisms with cysteine incorporated in medium and basic phenylmercuric nitrate (PMN) applied to filter paper discs*

PMN CONC. (M)	CYSTEINE CONC. (M)	INHIBITION ZONE, MM* 18 HR		
		<i>E. coli</i>	<i>E. typhosa</i>	<i>S. aureus</i>
$1.5 \times 10^{-4}$	0	21	23	31
$1.5 \times 10^{-5}$	0	17	0	23
$1.5 \times 10^{-6}$	0	0	0	19
$1.5 \times 10^{-4}$	$1.5 \times 10^{-6}$	19	19	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-6}$	0	0	23
$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	0	0	17
$1.5 \times 10^{-4}$	$1.5 \times 10^{-5}$	19	17	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-5}$	0	0	23
$1.5 \times 10^{-6}$	$1.5 \times 10^{-5}$	0	0	16
$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	17	17	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	0	0	23
$1.5 \times 10^{-6}$	$1.5 \times 10^{-4}$	0	0	16
$1.5 \times 10^{-4}$	$3.0 \times 10^{-4}$	16	19	31
$1.5 \times 10^{-5}$	$3.0 \times 10^{-4}$	0	0	23
$1.5 \times 10^{-6}$	$3.0 \times 10^{-4}$	0	0	16
$1.5 \times 10^{-4}$	$6.0 \times 10^{-4}$	16	17	31
$1.5 \times 10^{-5}$	$6.0 \times 10^{-4}$	0	0	23
$1.5 \times 10^{-6}$	$6.0 \times 10^{-4}$	0	0	15

\* Filter disc diameter, 12.7 mm. A 13-mm zone of inhibition therefore represents only trace activity, and less than this is represented in tables as 0 inhibition.

*Liquid medium.* Nutrient broth was prepared containing both phenylmercuric nitrate and cysteine in the same concentrations as were used in the experiments with the solid media. The inoculum was prepared by diluting 0.1 ml of a 48-hour broth culture of the test organism in 500 ml of sterile physiological saline and using 0.1 ml of this diluted culture to seed the tubes. Growth was determined after 18 hours' incubation at 37.5 C by centrifugation in Hopkins' tubes at 2,000 rpm for 20 minutes.

All experiments, employing both solid and liquid media, were accompanied by controls containing the sulfur compounds in the appropriate concentrations

TABLE 2

*Growth of organisms with cysteine, homocysteine or glutathione, and basic phenylmercuric nitrate (PMN) mixed and applied to filter paper disc*

PMN CONC. (M)	ANTAGONIST CONC. (M)	INHIBITION ZONE, MM,* 18 HR		
		<i>E. coli</i>	<i>E. typhosa</i>	<i>S. aureus</i>
Cysteine†				
$1.5 \times 10^{-4}$	0	23	23	31
$1.5 \times 10^{-5}$	0	17	0	21
$1.5 \times 10^{-6}$	0	0	0	17
$1.5 \times 10^{-4}$	$1.5 \times 10^{-6}$	23	23	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-6}$	15	0	21
$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	0	0	15
$1.5 \times 10^{-4}$	$1.5 \times 10^{-5}$	20	21	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-5}$	14	0	19
$1.5 \times 10^{-6}$	$1.5 \times 10^{-5}$	0	0	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	20	19	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	0	0	18
$1.5 \times 10^{-6}$	$1.5 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$3.0 \times 10^{-4}$	18	17	29
$1.5 \times 10^{-5}$	$3.0 \times 10^{-4}$	0	0	18
$1.5 \times 10^{-6}$	$3.0 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$6.0 \times 10^{-4}$	17	16	28
$1.5 \times 10^{-5}$	$6.0 \times 10^{-4}$	0	0	17
$1.5 \times 10^{-6}$	$6.0 \times 10^{-4}$	0	0	0
Homocysteine				
$1.5 \times 10^{-4}$	0	25	25	31
$1.5 \times 10^{-5}$	0	17	0	23
$1.5 \times 10^{-6}$	0	0	0	17
$1.5 \times 10^{-4}$	$1.5 \times 10^{-6}$	25	25	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-6}$	17	0	23
$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	0	0	15
$1.5 \times 10^{-4}$	$1.5 \times 10^{-5}$	25	23	31
$1.5 \times 10^{-5}$	$1.5 \times 10^{-5}$	15	0	21
$1.5 \times 10^{-6}$	$1.5 \times 10^{-5}$	0	0	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	23	19	29
$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	15	0	21
$1.5 \times 10^{-6}$	$1.5 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$3.0 \times 10^{-4}$	23	19	27
$1.5 \times 10^{-5}$	$3.0 \times 10^{-4}$	0	0	19
$1.5 \times 10^{-6}$	$3.0 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$6.0 \times 10^{-4}$	19	17	27
$1.5 \times 10^{-5}$	$6.0 \times 10^{-4}$	0	0	19
$1.5 \times 10^{-6}$	$6.0 \times 10^{-4}$	0	0	0

TABLE 2—Continued

PMN CONC. (M)	ANTAGONIST CONC. (M)	INHIBITION ZONE MM; 18 HR		
		<i>E. coli</i>	<i>E. typhosa</i>	<i>S. aureus</i>
Glutathione				
$1.5 \times 10^{-4}$	0	24	21	29.5
$1.5 \times 10^{-5}$	0	14	0	22.0
$1.5 \times 10^{-6}$	0	0	0	15.0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-6}$	24	21	29
$1.5 \times 10^{-5}$	$1.5 \times 10^{-6}$	14	0	21
$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	0	0	15
$1.5 \times 10^{-4}$	$1.5 \times 10^{-5}$	24	20	29
$1.5 \times 10^{-5}$	$1.5 \times 10^{-5}$	13	0	16
$1.5 \times 10^{-6}$	$1.5 \times 10^{-5}$	0	0	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	24	18	29
$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	13	0	16
$1.5 \times 10^{-6}$	$1.5 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$3.0 \times 10^{-4}$	22	16	26
$1.5 \times 10^{-5}$	$3.0 \times 10^{-4}$	0	0	15
$1.5 \times 10^{-6}$	$3.0 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-4}$	$6.0 \times 10^{-4}$	20	15	25.5
$1.5 \times 10^{-5}$	$6.0 \times 10^{-4}$	0	0	14.0
$1.5 \times 10^{-6}$	$6.0 \times 10^{-4}$	0	0	0

\* Filter disc diameter, 12.7 mm. A 13-mm zone of inhibition therefore represents only trace activity and less than this is represented in tables as 0 inhibition.

†  $7.5 \times 10^{-5}$  M PMN was completely antagonized for *E. coli* by  $6.0 \times 10^{-4}$  M cysteine and for *E. typhosa* by  $1.2 \times 10^{-3}$  M cysteine (control zones, 19 mm).  $1.5 \times 10^{-5}$  M PMN was completely antagonized for *S. aureus* by  $1.2 \times 10^{-3}$  M cysteine.

and omitting the mercurial. In the concentrations used, the compounds thus added did not affect the growth of the bacteria.

The test organisms were *Escherichia coli* ATCC no. 730, *Eberthella typhosa* ATCC no. 7251, and *Staphylococcus aureus*, ATCC no. 152.

The basic phenylmercuric nitrate ( $C_6H_5HgNO_3 \cdot C_6H_5HgOH$ ) was obtained from The Hamilton Laboratories, Inc., glutathione from the Schwarz Laboratories Inc., and *l*(+)-cysteine hydrochloride, *l*(-)-cystine, *dl*-homocysteine, and *dl*-methionine from General Biochemicals, Inc.

#### RESULTS

The data in table 1 demonstrate an antagonism between cysteine in the medium and phenylmercuric nitrate on the filter paper disc as shown by the diminution in the zones of inhibition surrounding the discs. As mentioned earlier, this method does not permit comparison of effective concentrations of



the germicide and cysteine and was abandoned in favor of dipping the discs in mixtures of the germicide and —SH compound. In table 2 are shown the effects by this method of the sulfhydryl-containing compounds cysteine, homocysteine, and glutathione on the growth-inhibiting activity of phenylmercuric nitrate. There is a marked difference in the sensitivity of the individual test organism to the germicide as shown by the zones of inhibition in the control group. The greater sensitivity of *Staphylococcus aureus* to the mercurial is in conformity with the findings of Weed and Ecker (1931) and Birkhaug (1933). Within

TABLE 3

*Growth of organisms with cysteine and basic phenylmercuric nitrate (PMN) in nutrient broth*

PMN CONC. (M)	CYSTEINE CONC. (M)	GROWTH, ML HOPKINS' TUBES, 18 HR		
		<i>E. coli</i>	<i>E. typhosa</i>	<i>S. aureus</i>
0	0	0.023	0.002	0.002
$1.5 \times 10^{-4}$	0	0	0	0
$1.5 \times 10^{-5}$	0	0.013	0.001	0
$1.5 \times 10^{-6}$	0	0.023	0.002	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-6}$	0	0	0
$1.5 \times 10^{-5}$	$1.5 \times 10^{-6}$	0.010	0.001	0
$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	0.023	0.002	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-5}$	0	0	0
$1.5 \times 10^{-5}$	$1.5 \times 10^{-5}$	0.012	0.001	0
$1.5 \times 10^{-6}$	$1.5 \times 10^{-5}$	0.023	0.002	0
$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	0.010	0.001	0
$1.5 \times 10^{-6}$	$1.5 \times 10^{-4}$	0.023	0.002	0.002
$1.5 \times 10^{-4}$	$3.0 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-5}$	$3.0 \times 10^{-4}$	0.014	0.002	0
$1.5 \times 10^{-6}$	$3.0 \times 10^{-4}$	0.023	0.002	0.002
$1.5 \times 10^{-4}$	$6.0 \times 10^{-4}$	0	0	0
$1.5 \times 10^{-5}$	$6.0 \times 10^{-4}$	0.018	0.002	0.001
$1.5 \times 10^{-6}$	$6.0 \times 10^{-4}$	0.023	0.002	0.002

experimental error, the sulfhydryl-containing compounds are equally effective in overcoming the action of the germicide. In contrast, the compounds in which the —SH group is covered (cystine and methionine) were found to be ineffective, and therefore the results of experiments with these compounds have not been tabulated.

In table 3 is demonstrated the action of cysteine and phenylmercuric nitrate on the growth of the organisms in nutrient broth. Again, antagonism is evident.

#### DISCUSSION

These experiments show that phenylmercuric nitrate inhibition of the growth of *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* can be dimin-

ished or prevented by the combination of the germicide with the sulfhydryl-containing compounds cysteine, glutathione, and homocysteine, but not with cystine and methionine.

The reaction between the —SH group and the germicide can be demonstrated, since the nitroprusside test for the sulfhydryl group becomes negative in the test tube when the molar ratio of sulfhydryl compound:germicide is greater than 2:1, as required by theory for the reaction with both mercury atoms of basic phenylmercuric nitrate ( $\text{C}_6\text{H}_5\text{HgNO}_3 \cdot \text{C}_6\text{H}_5\text{HgOH}$ ).

In the filter disc experiments, the sulfhydryl compounds at a concentration of  $6.0 \times 10^{-4}$  M did not completely overcome the effects of the highest concentration of phenylmercuric nitrate ( $1.5 \times 10^{-4}$  M), although the germicidal effectiveness of this concentration of mercurial was reduced for all organisms. The inhibition of *Eberthella typhosa* by  $7.5 \times 10^{-5}$  M phenylmercuric nitrate was offset completely by  $1.2 \times 10^{-3}$  M cysteine. Inhibition of *Escherichia coli* by  $1.5 \times 10^{-5}$  M phenylmercuric nitrate could be completely offset by  $3.0 \times 10^{-4}$  M or greater concentrations of the sulfhydryl compounds. *Staphylococcus aureus* was the most sensitive of the organisms to the germicide, and it was possible to obliterate the activity of  $1.5 \times 10^{-6}$  M phenylmercuric nitrate by  $1.5 \times 10^{-5}$  M or greater concentrations of the sulfhydryl compounds. Thus, in the filter disc determinations, 10 or more moles (5 or more equivalents) of sulfhydryl compound per mole of phenylmercuric nitrate were required for suppression of activity. In nutrient broth, from 20 to 100 moles of cysteine per mole of mercurial (10 to 50 equivalents) were necessary to secure antagonism.

In the work on yeast respiration and on enzymes (Cook, Perisutti, and Walsh, 1946; Cook and Perisutti, 1947; and unpublished data) it was also observed that concentrations of sulfhydryl compounds several times greater than the theoretical were required for the antagonism of phenylmercuric nitrate. Fildes (1940) and Cavallito, Bailey, Haskell, McCormick, and Warner (1945) made similar observations for the antagonism of  $\text{HgCl}_2$  toxicity by sulfhydryl compounds.

These findings suggest that the organic mercurial, basic phenylmercuric nitrate, like mercuric chloride and certain of the natural antibiotics, may be presumed to react with essential —SH groups in the microorganism, supposedly in enzyme systems. Although such action probably occurs, as in the inhibition of succinic dehydrogenase, the previously observed inhibition by phenylmercuric nitrate of such enzymes as cytochrome oxidase and catalase (Cook, Kreke, McDevitt, and Bartlett, 1946), which have been shown not to require —SH groups for their functioning (Barron and Singer, 1945), and the inability of sulfhydryl compounds to reverse the depression of cytochrome oxidase and yeast respiration by phenylmercuric nitrate (Cook and Perisutti, 1947) have suggested that the germicide may not be specific for —SH groups, but may also react with other active groups in the enzyme protein. Consonant with these suggestions is the failure of attempts by the present authors to reverse the inhibiting effects of phenylmercuric nitrate on *Escherichia coli* in broth by subsequent addition (after 0.5 to 6 hours) of as much as 50 equivalents of cysteine or glutathione. The reversal experiments, however, leave much to be desired

since the high concentrations of sulfhydryl compounds (above  $1.5 \times 10^{-3}$  M) in themselves were found to be inhibitory to the organisms. Sahyun *et al.* (1936) also reported that high concentrations of cysteine inhibited the growth of *Escherichia coli* in a synthetic medium. Other evidence suggests that phenylmercuric nitrate may be firmly bound to yeast cells. For example, it was impossible to reverse the respiratory depressing effects of the mercurial on yeast respiration with yeast extract (Cook and Kreke, 1943), nor could the respiration of yeast be restored by washing the phenylmercuric nitrate from the cells after a 15-minute exposure (Cook and Perisutti, unpublished).

#### SUMMARY

The growth-inhibiting action of basic phenylmercuric nitrate on *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* can be antagonized by the sulfhydryl-containing compounds cysteine, homocysteine, and glutathione, but not by cystine and methionine.

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# A BACTERIAL VIRUS FOR ACTINOMYCES GRISEUS<sup>1</sup>

H. B. WOODRUFF, T. D. NUNHEIMER, AND S. B. LEE

*Research Laboratories of Merck and Co., Inc., Rahway, New Jersey, and Elkton, Virginia*

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Attack by bacterial viruses on members of the genus *Actinomyces* has been reported in only a few instances, but bacteriophages which attack *Eumycetes* are prevalent. Many industrial processes which employ bacteria are subject to bacteriophage infestations. The isolation of bacteriophage from soil and sewage may be easily accomplished, but soil cannot be considered an abundant source of actinophage. Isolations of actinomycetes are usually made from fertile soils; however, evidence of phage action is seldom noted. A transmissible and filterable lytic agent, which attacks actinomycetes, was reported by Wieringa and Wiebols (1936). This particular phenomenon may be explained as being due to the action of a polyvalent actinophage which initiated lysis not only of the parent culture but also of several other species. There are other reports of lysis of *Actinomyces* for which actinophages could not be demonstrated. However, the methods used would fail to demonstrate the actinophage for *Actinomyces griseus* (Dmitrieff and Soutéeff, 1936; Katznelson, 1940).

Accompanying the recent large-scale industrial utilization of actinomycetes for the production of antibiotic substances, study of the group has been intensified (Schatz, Bugie, and Waksman, 1944; Porter, 1946). The accumulative generations of growth of the cultures, constantly subject to chance contamination through faulty air filtration or insufficiently sterile laboratory and plant equipment and through errors in techniques, have made it highly probable that actinophages would be rediscovered for actinomycetes. In fact, a recent report indicated that an actinophage has been isolated from the streptomycin fermentation (Saudek and Colingsworth, 1947).

## EXPERIMENTAL WORK

We have observed an actinophage in laboratory cultures of *A. griseus* which were exposed to laboratory air for a 24-hour period. Moreover, outbreaks have occurred in a streptomycin production plant, located about 500 miles distant from the research laboratory. First recognition of the actinophage occurred in laboratory shake flasks. *A. griseus* cultures, which had developed under submerged conditions for 24 hours from a 10 per cent vegetative inoculum, were changed to stationary incubation conditions and the cotton plugs removed. Thin pellicles developed which showed evidence of plaque formation similar to that usually associated with bacteriophage development. The cultures were

<sup>1</sup> Throughout this paper the designation *Actinomyces* has been used to conform with the fifth edition of *Bergey's Manual of Determinative Bacteriology*. In each case, the organism referred to may be classified under the terminology proposed by Waksman and Henrici (1943) as *Streptomyces*.

filtered through ultrafine fritted glass filters, and the filtrates proved to be free from bacterial or actinomycete contamination. The filtrate, when added to a newly inoculated submerged culture of *A. griseus*, prevented initiation of growth.

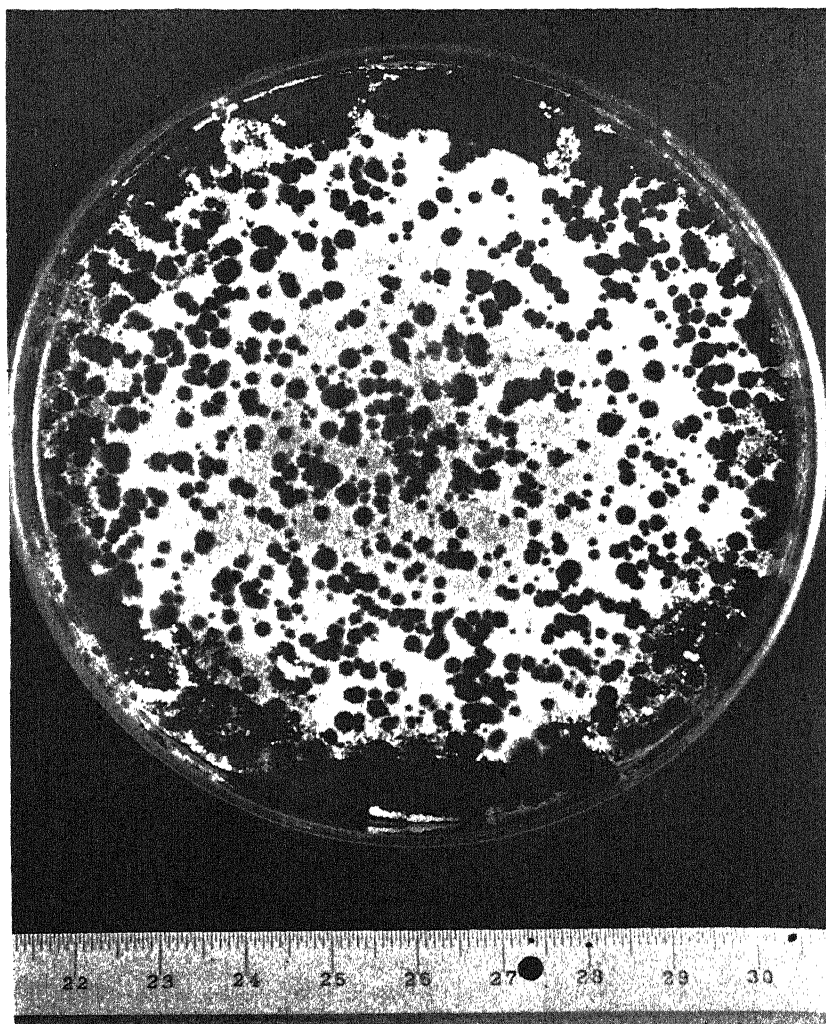


FIG. 1. THE FORMATION OF PLAQUES ON A PETRI DISH CULTURE

Various dilutions of the filtrate were placed on an agar medium with *A. griseus* spores. The typical "moth-eaten" cultures, characteristic of bacteriophage contamination, developed within 24 hours. Plaques did not spread during additional incubation. Within 48 hours the *A. griseus* growth between the plaques had sporulated and counts of the plaques could be made with ease

(figure 1). The filtrate from the culture in which the first evidence of actinophage was noted contained 55,000,000 plaque-forming units per ml. A few resistant cultures of *A. griseus* developed when exposed to high concentrations of the actinophage.

Actinophage infestations of *A. griseus* in a streptomycin production plant have occurred. In each case simultaneous bacterial contamination or other factors indicated an outside source of the actinophage. No evidence has been found that the actinophage was derived from stock cultures of *A. griseus*.

*Multiplication of actinophage.* The lytic agent was carried through several cultures of *A. griseus* in series and initiated lysis in each instance. To prove transmissibility of the agent, 0.01 ml of a bacteria-free filtrate was transferred to 50 ml of *A. griseus* culture. After 24 hours of submerged growth, the lysed culture was filtered and 0.01 ml of filtrate added to a new culture. The transfers, with filtrations between each, were continued for a total of six cultures.

TABLE 1  
*Multiplication of actinophage*

TRANSFER	ACTINOPHAGE PER ML	MULTIPLICATION FACTOR	
		Individual transfers	Accumulative
Phage inoculum.....	20,000,000,000		
1st transfer.....	32,800,000,000	8,200	8,200
2nd transfer.....	100,000,000,000	16,000	$131 \times 10^6$
3rd transfer.....	36,000,000,000	1,800	$236 \times 10^9$
4th transfer.....	48,000,000,000	6,600	$156 \times 10^{13}$
5th transfer.....	64,000,000,000	6,600	$103 \times 10^{17}$
6th transfer.....	9,600,000,000	735	$75 \times 10^{20}$
Control <i>A. griseus</i> .....	<10		

Filtrates from each flask were saved and plated by the plaque method for the determination of numbers of actinophage. These determinations (table 1) prove that the agent is transmissible and multiplies after each transfer. For each plaque-forming particle added to the first *A. griseus* culture in the series, a total of  $75 \times 10^{20}$  particles had been produced on completion of the sixth transfer.

*Actinophage-susceptible strains of A. griseus.* Most bacteriophages are specific in activity against a single strain of a species. Actinophage was first isolated from cultures of *A. griseus* no. 9, from the collection of the New Jersey Agricultural Experiment Station, and was subsequently found in fermentations with other strains, of different streptomycin-producing capacities, from the collection. Likewise, three ultraviolet mutants of *A. griseus*, morphologically distinct from the parent, were susceptible. Centraalbureau voor Schimmelcultuur cultures labeled *A. griseus* Waksman and Curtis and *A. griseus* Bucherer were resistant to the action of the actinophage. However, no streptomycin was produced by these strains. Six additional species of *Actinomyces* were not affected by the actinophage.

*Effect of culture age.* The actinophage multiplies at the expense of submerged cultures of *A. griseus* of various ages. Complete lysis has been noted only with an inoculum consisting of spores of *A. griseus*. Six hours after inoculation the cultures incubated with actinophage show a faint turbidity due to germinated spores. Shortly thereafter, the cultures lyse completely, and only occasionally does a resistant colony grow out. With submerged vegetative inoculum, actinophage multiplication can be proved by the determination of numbers by the plaque method, but lysis is not complete. With 5 to 10 per cent by volume of submerged inoculum, there is little difference in degree of turbidity and streptomycin production in 24-hour-old control cultures and in cultures infected with actinophage. Usually, the infected cultures fragment at an earlier time than control cultures. Since streptomycin accumulation ceases about the time of fragmentation, yields are lower in infected cultures. The majority of *A. griseus* cells which remain in the infected cultures following fragmentation are resistant

TABLE 2  
*Sensitivity of bacteriophages to chemicals in the absence of cells*

AGENT*	VIRICIDAL DILUTION	
	<i>E. coli</i> bacteriophage	<i>A. griseus</i> actinophage
Acriflavine.....	<0.004 mg/ml	0.004 mg/ml
<i>Actinomyces</i> 34.....	1:250	1:32
<i>Actinomyces</i> 11.....	1:65	<1:2
<i>Bacterium</i> 24.....	1:250	<1:2

\* Bacterial virus exposed to agent 16 hours at 37 C in nutrient broth substrate.

to the action of the actinophage. Of 13 production lots of *A. griseus* which fragmented early, 11 were found to contain actinophage.

*Actinophage-resistant strains of A. griseus.* Several resistant cultures have been selected following exposure of *A. griseus* to the actinophage. Approximately half of the isolates are equal to the parent in streptomycin production. Many appear to be lysogenic. One culture, which produced high yields of streptomycin in the presence or absence of added actinophage, always had two or three plaques of lysis in agar slant cultures. Filtrates of a series of four submerged culture transfers, in series, all contained approximately 100 plaque-forming actinophage particles per ml for a sensitive strain of *A. griseus*. The actinophage was capable of multiplying to a slight extent on the resistant isolate. Such cultures are dangerous for routine use in the production of streptomycin, since conditions are favorable for the multiplication of any actinophage variants which gain the ability to attack the resistant culture.

*Sensitivity of actinophage to heat.* The susceptibility of the actinophage to heat was determined. Filtrates of a lysed culture of *A. griseus* grown on a glucose "N-Z-amine" meat extract medium were used as a source of actinophage. No evidence of destruction occurred from heating a filtrate containing 2,880,-



000,000 particles per ml at 80 C for 15 minutes in a water bath. At 85 C, 0.02 per cent of the actinophage particles survived, and at 90 C, 0.00002 per cent survived. Only 0.5 per cent of 500,000,000 *A. griseus* spores per ml of water remained viable after heating at 60 C for 15 minutes.

*Sensitivity of actinophage to chemicals.* Several compounds have been shown to destroy *Escherichia coli* bacteriophage during a 16-hour incubation at 24 C in nutrient broth, in the absence of bacterial cells. Acriflavine, a filtrate from an unidentified bacterium, and filtrates from two actinomycetes have been most active. The agents were tested against the actinophage under similar conditions. The latter virus was more resistant than the *E. coli* bacteriophage (table 2).

*A. griseus* was inhibited by acriflavine in concentrations which were destructive to the actinophage. The filtrate of *Actinomyces* 34, which had no

TABLE 3  
*Sensitivity of bacteriophages to chemicals in the presence of cells*

AGENT	<i>E. coli</i> PER ML*		MG <i>A. griseus</i> CULTURE†	
	Control	+ Phage	Control	+ Phage
None.....	$3 \times 10^{11}$	$2 \times 10^3$	50	7
1:40 Act. no. 34.....	$2 \times 10^{11}$	$3 \times 10^8$	43	8
0:001 mg/ml Acriflavine.....	$2 \times 10^{11}$	$5 \times 10^6$	4	7
0:0001 mg/ml Acriflavine.....	$1 \times 10^{11}$	$2 \times 10^3$	1	3

\* Four hours' incubation.

† Twenty-four hours' incubation from spore inoculum, 30 ml volume.

inhibitory effect on growth of *A. griseus*, did not retard lysis of *A. griseus* by the actinophage (table 3).

*Morphology of actinophage.*<sup>2</sup> Preparations made from cover slip impressions of plaques for electron microscope observation demonstrated the particulate nature of the lytic agent and its close resemblance to strains of *E. coli* bacteriophage (Luria and Anderson, 1942). The chromium shadowing technique indicated a surprising diversity of structure of the actinophage particles (figures 2 and 3). Practically all particles had a long, relatively thick but bent tail of approximately 0.015 by 0.15 microns. Whereas the majority of the heads appeared symmetrically spherical, 0.05 microns in diameter, many were composed of two distinct bodies and a few appeared to be similar to tetrads.

One or two preparations had a majority of particles with two tails (figure 3). The heads did not appear sufficiently dense to indicate that these particles were simply overlying actinophages, and it seems possible that the preparations represented plaques formed by morphological variants of actinophage which

<sup>2</sup> Electron microscope studies were made by Dr. James Hillier in the laboratories of the Radio Corporation of America, Princeton, New Jersey, with preparations supplied from our laboratory.

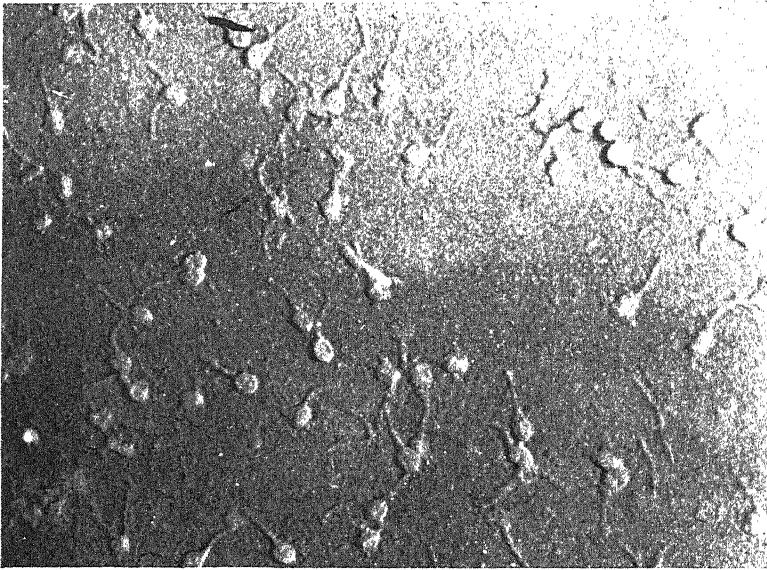


FIG. 2. ELECTRON MICROGRAPH SHOWING THE ACTINOPHAGE.  $\times 37,500$

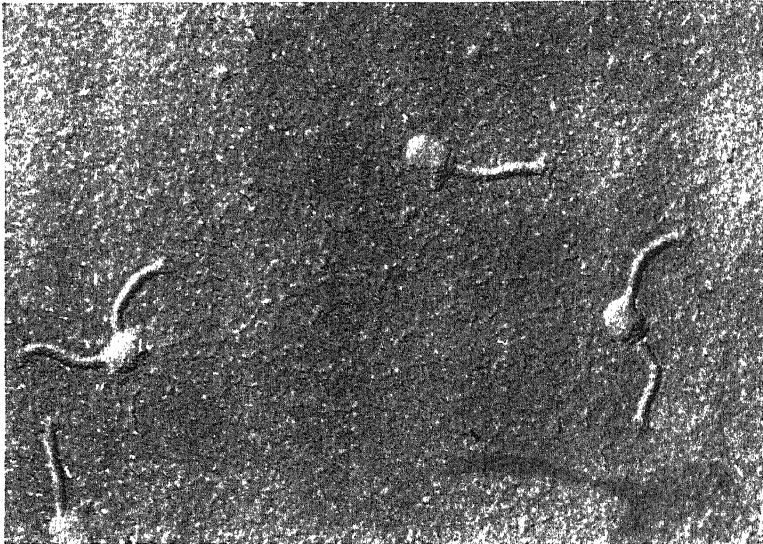


FIG. 3. ELECTRON MICROGRAPH SHOWING ACTINOPHAGE PARTICLES WHICH APPEAR TO HAVE TWO TAILS.  $\times 95,000$

retained infectivity. However, no proof can be offered at present for the origin of this unusual type. They were not present in the majority of the preparations.

#### SUMMARY

An actinophage has been isolated which infects strains of *Actinomyces griseus*. The virus is particulate, transmissible, and initiates lysis in young cells of *A. griseus*. It is more resistant to heat than are the spores of *A. griseus*, but is susceptible to certain viricidal agents which destroy *Escherichia coli* bacteriophage. Resistant cultures of *A. griseus* have been developed which may be lysogenic. Electron micrographs prove the particulate nature and demonstrate the morphology of the actinophage.

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## NOTE

### THE RAPID RECOGNITION OF ASPERGILLIC ACID

WALTER C. TOBIE AND CLARA ALVERSON

*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

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In examining unidentified microorganisms from natural sources in a search for new antibiotics, it is necessary to exclude previously known antibiotics. This is usually done by running a so-called "bacterial spectrum" against a number of different test organisms which differ in sensitivity to the action of known antibiotic substances. However, even when the presence or absence of a known antibiotic is determined provisionally by this biological method, it is usually necessary to obtain additional evidence by chemical methods. This may require a large amount of extra work.

An unidentified mold (our no. 401) gave strong antibiotic activity (wide zones of inhibition of growth on agar plates) with both gram-positive and gram-negative bacteria. When cultured for 7 days or longer at 25 C, in 100-ml lots of trypticase soy broth (Baltimore Biological Laboratories) in 500-ml Erlenmeyer flasks, the surface became completely covered by a white mycelium, with areas of gray-green spores. The initial pH of 6.8 increased to 7.7 or higher. Sterile filtrates neutralized to pH 7 also showed strong activity against bacteria. Bacterial spectra run in comparison with a known sample of aspergillic acid strongly suggested that the latter acid was present. It was noted that *Pasteurella multocida*, Lederle strain, was inhibited by aspergillic acid in much higher dilutions than the other test organisms, making it a useful test organism in testing for this acid.

In 1943 Menzel, Wintersteiner, and Rake (J. Bact., **46**, 109) briefly mentioned that aspergillic acid is volatile with steam. This observation was applied by evaporating or distilling the alkaline broth cultures to about a third of the original volume. The concentrate was adjusted to pH 4.2 with HCl. Upon rapid distillation, a pale yellow, waxy-appearing, amorphous solid separated in the condenser and receiver. On standing overnight, more material separated in a microcrystalline form. The solid was dissolved out of the aqueous suspension, using chloroform in a separatory funnel. Evaporation of the extract in a current of air at room temperature yielded a viscous yellow residue which slowly hardened to a crystalline mass. The material was identified by means of its infrared spectrum, which agreed with that of aspergillic acid in all respects.

Distillation of broth cultures adjusted to pH 4.2 without preliminary concentration gave a solution of aspergillic acid from which no solid separated. However, the presence of the acid was demonstrated by adding 1 drop of reagent to 3-ml portions of the distillate. A strong brown color was obtained

with 1 N  $\text{FeCl}_3$ , 1 per cent copper sulfate gave a voluminous pale green precipitate, and 1 per cent cobalt chloride gave a less abundant flesh-colored or pale orange precipitate or turbidity. The saturated solutions remaining after solid aspergillic acid had separated from the distillates of preconcentrated cultures, and solutions of known aspergillic acid, gave similar reactions.

These tests permit the recognition of aspergillic acid with a high degree of probability. The tests proved useful with a second mold (our no. 415). In this case it was possible to identify aspergillic acid with very little other work.

It is also obvious that distillation may be used as a step in the preparation of aspergillic acid. Distillation alone will not necessarily give a pure product, since several common fatty acids are appreciably volatile with steam. It should also be noted that substances are known to exist which are very similar to aspergillic acid but not identical with it. The volatility of these substances with steam is yet to be determined.

Acknowledgments are made to Dr. James D. Dutcher, Squibb Institute for Medical Research, who furnished a control sample of very pure aspergillic acid; to Dr. R. C. Gore and associates of our Physics Division, who ran and interpreted the infrared spectra; and to Dr. Kenneth B. Raper of the Northern Regional Research Laboratories, who identified our mold no. 401 as a typical strain of *Aspergillus flavus*.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## MICHIGAN BRANCH

DETROIT, MICHIGAN, JUNE 12, 1947

THE INCIDENCE OF ENTEROCOCCI IN HUMAN FECES. *Morris F. White, Joseph A. Kasper, and Elizabeth J. Cope.*

Fecal specimens received in the laboratory of the Detroit Department of Health served as the source of material studied in this investigation. The culture medium and methods as outlined by Winter and Sandholzer were adopted; however, the medium was modified to the extent that penicillin was omitted from the confirmation broth.

In this series of cultures there was a total of 200 fecal specimens examined. Positive presumptive findings were shown for all of the samples. Of the total number, 115 showed the confirmatory findings for the presence of enterococci, but 85 cultures were considered negative. Thus, organisms of the enterococcus group were recovered from 57.5 per cent of the cultures in this series.

The repeated failure to isolate enterococci from the feces of 7 persons in this study indicates that some humans may not be carriers of enterococci at all times.

These findings seemingly indicate that enterococci are not always present in the feces of humans. Implicit reliance upon the finding of enterococci as a single indicator of human fecal pollution of water

cannot, as yet, be accepted without question.

AN ACTINOPHAGE IN STREPTOMYCIN-PRODUCING CULTURES OF STREPTOMYCES GRISEUS. *R. M. Smith, W. H. Kuhn, and G. R. M. Miesel.*

An actinophage which affects cells of *Streptomyces griseus* has been found. Its presence has been noted in stock cultures and in fermentation beers of various types. Plaques typical of bacteriophage action were found when infected cultures were grown on agar media and lysis was noted in cultures grown by submerged methods. The lytic agent increases in quantity upon cultivation of the infected cultures, passes through Seitz and other bacteriological filters, and is relatively heat-stable.

Examination of stock cultures revealed that most of them were infected, and attempts were made to render cultures phage-resistant. Exposure of the susceptible cultures to the phage under various conditions resulted in the development of resistant strains. These strains, thus far, have shown no tendency to revert to susceptibility. The streptomycin-producing capacity of the strains which we have rendered resistant has not differed appreciably from that of the parent cultures.

## NORTHERN CALIFORNIA-HAWAIIAN BRANCH

STANFORD UNIVERSITY, CALIFORNIA, JUNE 14, 1947

SELECTIVE BLOOD FACTORS AFFECTING BACTERIAL VARIATION. *Werner Braun*, Division of Veterinary Science, University of California, Berkeley, California.

The selective factor suppressing the establishment of nonsmooth variants of *Brucella abortus*, previously demonstrated in normal serum of various *Brucella*-susceptible animals, has been found in the gamma globulin fraction. *In vivo*, modifications of

the gamma globulin, which occur after vaccination, alter the selective activity of normal gamma globulin. *In vitro*, preliminary tests have indicated that the selective activity of normal gamma globulin disappears in the presence of sufficient anti-gamma globulin (produced by inoculation of bovine gamma globulin into rabbits). Similarly, in the presence of high albumin concentrations, corresponding to approximately twice

the normal blood concentrations, gamma globulin fails to express its selective activity. It is hoped that this information will lead to the creation of *in vivo* conditions which will favor the establishment of non-smooth, avirulent variants.

**PENICILLIN STABILITY IN PHOSPHATE, ACETATE, AND CITRATE BUFFERS.** *John O. Thomas*, Biological Research Department, Cutter Laboratories, Berkeley, California.

The stability of crystalline potassium penicillin G (1,530 units per mg) in  $\text{NaH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffers (pH 6.0), of final molarities M/16, M/50, M/100, and M/200, and in M/50 acetate and M/50 citrate buffers was studied for a maximum of 86 days, the initial potencies of the sterile mixtures being approximately 10,000 units per ml. Sealed 5-ml volumes of each mixture were kept at 37, 24, and 2 C, one set of mixtures in a temperature group being cup-assayed against *Staphylococcus aureus* (NRRL 318), and the pH's being measured, on a particular day. Residual activities were computed as percentages of zero time potencies.

Penicillin destruction at 37 C was rapid, first-order curves resulting. Similar less steep curves were encountered at 24 C. At both temperatures protection efficiency followed buffer capacity, with the exception of citrate, which was the most efficient.

At 2 C, a first-order inactivation curve resulted for the saline control. The buffered mixtures' curves, however, all showed periods, from 10 days (M/100 phosphate) to 72 days (acetate), when the activities did not drop below 100 per cent. These indicate activity potentiation because maximal potencies, for example, of 150 per cent and 138 per cent (assay error about 10 per cent) occurred in the acetate and M/200 phosphate buffers, respectively, and these in spite of corresponding pH drops to 5.50 and 5.30.

Except for saline and M/200 phosphate, all 2 C curves showed an initial rise, a moderate fall, and a second rise before final drops, the rises being independent of pH drops, though pH's remained practically constant in citrate and M/16 phosphate. No second rise occurred in M/200 phosphate, the pH of which (4.70) was the lowest of the buffers, at 50 days. Acetate provided the best protec-

tion, despite a pH fall to 5.20 at 86 days. The buffer ions are apparently concerned with these phenomena.

**AN IMPROVED TECHNIQUE FOR BACTERIOLOGICAL CULTURE STUDIES.** *Phillip J. Brady and Paul Esau*, Research Laboratories, California Packing Corporation, San Francisco, California.

A simple, convenient, and inexpensive double compartment culture tube for fermentations and aerobic and anaerobic culture studies has been designed. Its uses can be enumerated as follows:

(1) A liquid medium or agar is put in the long arm and pyrogallol in the short arm of the tube. Anaerobes can be cultured by closing the tube with a rubber stopper after a cotton plug.

(2) The nature of a gas produced by bacteria (usually  $\text{CO}_2$ ) can be detected by putting lime water (filtered) in the short arm. Precipitation of calcium carbonate designates  $\text{CO}_2$ .

(3) Partial neutralization of acid media by hydrolysis during sterilization is avoided by placing the neutral medium in the long arm and the acid medium in the short arm and mixing together after autoclaving and cooling.

(4) Carbohydrate media for fermentation studies can be prepared by placing the sugar solution in the short arm, and the peptone broth with indicator and gas vial in the long arm. After being autoclaved for 10 to 15 minutes at 10 to 15 pounds' pressure, the medium is cooled and the ingredients combined in the long arm. The short arm can now be used for the detection of gas or for creating anaerobic conditions.

**DECOMPOSITION OF TARTRATES BY SOME MESOPHILIC, SPOREFORMING, OBLIGATE ANAEROBES.** *Joseph Tabachnick*, Division of Food Technology, University of California, Berkeley, California.

Since the classical experiment performed by Pasteur in 1863 in which he demonstrated the existence of obligate anaerobes (with calcium tartrate as a substrate), very little work has been done with the obligatory anaerobic bacteria which decompose tartrate. None of the later investigations were made with pure cultures.



Twenty-three strains of tartrate-fermenting clostridia were isolated by an enrichment technique from calcium tartrate recovery equipment and spoiled calcium tartrate, as well as from soils.

With the exception of their ability to utilize glycerol and tartrate, the majority of the strains isolated were closely related to the type species, *Clostridium butyricum*, as described in Bergey *et al.* (1939). Glucose was fermented with the production of carbon dioxide, hydrogen, butyric and acetic acids, and very small amounts of neutral volatile products. Tartrate was fermented

with the production of carbon dioxide, hydrogen, and acetic acid, and small amounts of butyric acid and ethanol. Trace amounts of pyruvic acid from the tartrate fermentation were isolated and identified.

With the exception of *l*-malic acid, four carbon dicarboxylic acids other than *d*-tartaric acid were not attacked by the cultures investigated.

The enzymes involved in the decomposition of tartrate were shown to be adaptive in character. Attempts to adapt other cultures of the common saccharolytic clostridia to the utilization of tartrate were unsuccessful.



# STUDIES ON POLYMYXIN: ISOLATION AND IDENTIFICATION OF *BACILLUS POLYMYXA* AND DIFFERENTIATION OF POLYMYXIN FROM CERTAIN KNOWN ANTIBIOTICS

P. G. STANSLY AND M. E. SCHLOSSER

*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

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Polymyxin is an antibiotic substance occurring in the culture filtrates of *Bacillus polymyxa*. The isolated substance is unique in its specificity for gram-negative bacteria. A summary of the more important results obtained during the course of several years, including chemotherapeutic and toxicity data, has been reported (Stansly, Shepherd, and White, 1947). The present contribution is concerned with the isolation and identification of the antibiotic-producing organism and some early findings which both characterized and distinguished polymyxin from certain known antibiotics.

*Isolation of Bacillus polymyxa.* *Bacillus polymyxa* was isolated from soil in the course of a program designed to find new antibiotics for the chemotherapy of gram-negative bacterial infections. The test organism used in this search was *Salmonella schottmuelleri*. Our method for isolating antibiotic-producing organisms with a specific type of activity involves the preparation of pour plates of soil dilutions using a variety of media and cultural conditions. The plates are subsequently sprayed with a suspension of the test organism by means of an apparatus designed for the purpose (Stansly, 1947).

*Identification of Bacillus polymyxa.* The identification of *Bacillus polymyxa* was established by following the key to the identification of aerobic sporeforming bacteria by Smith, Gordon, and Clark (1946). In the preliminary work,<sup>1</sup> edition 5 of Bergey's *Manual of Determinative Bacteriology* (1939) and the galley proofs of edition 6 were found helpful.

An 18-hour broth culture consisted of gram-negative rods with few or no gram-positive cells. Older cultures showed vegetative cells and oval spores either free or central to terminal in adhering and swollen sporangia. Broth cultures at 30 C were turbid and had a ropy sediment. Indole was not formed. Nitrates were reduced to nitrites. Hydrogen sulfide was not produced. Acid and gas were formed from glucose, lactose, and sucrose. Acid but no gas was produced from rhamnose and a slight amount of acid but no gas from sorbitol. Starch was hydrolyzed. Acid and gas were produced from litmus milk, which was coagulated and reduced.

The existence of oval spores, central to terminal, and sporangia frequently adhering and swollen, plus the predominant gram-negative nature of the vegeta-

<sup>1</sup> The authors are indebted to Dr. Walter C. Tobie and Miss Marion H. Cook for the preliminary work which led to the conclusion that the antibiotic-producing organism had characteristics intermediate between those of *Bacillus polymyxa* and *Bacillus macerans*.

tive forms, placed the organism in group 2 in the classification of Smith, Gordon, and Clark. The fermentation of carbohydrates, such as glucose, lactose, and sucrose, with the formation of both acid and gas narrowed the possible identity of the organism to one of two species, namely, *Bacillus polymyxa* or *Bacillus macerans*. These two species may be distinguished in the following ways: (1) *B. polymyxa* produces acetylmethylcarbinol from the proper substrate, whereas *B. macerans* does not; (2) *B. polymyxa* does not produce an amylase which catalyzes the formation of crystalline dextrans from starch, whereas *B. macerans* produces this enzyme. Both of these criteria were used to identify the unknown organism.<sup>2</sup>

*Production of acetylmethylcarbinol.* Three known *B. polymyxa* strains (ATCC nos. 8523, 7047, and 7070), one *B. macerans* (ATCC no. 355), and the unidentified organism were inoculated in the recommended neopeptone medium and under the suggested conditions (Smith *et al.*, 1946). The test for acetylmethylcarbinol was made according to O'Meara (1931). *B. macerans* was negative for acetylmethylcarbinol on the third, fifth, seventh, and fourteenth day of incubation, whereas the isolated organism and the three *polymyxa* strains were positive at these times.

*Formation of crystalline dextrans.* The formation of crystalline dextrans from starch was detected by the iodine test of Tilden and Hudson (1942). The same strains of *B. polymyxa* and *B. macerans* were used as before, in a medium and under conditions recommended (Smith *et al.*, 1946), with the exception that Merck's soluble starch was used instead of Takamine or White Rose. *B. macerans* gave a positive test for crystalline dextrans (both hexagons and needles were observed) when tested after 2 weeks and again after 3 weeks of incubation. All three *polymyxa* strains and the antibiotic-producing organism were negative at these times.

The two foregoing critical tests supported each other in identifying the organism as a strain of *Bacillus polymyxa*, a species apparently first described in 1880 as *Clostridium polymyxa* (Smith *et al.*, 1946) and of current interest in the production of 2,3-butanediol by fermentation (Adams, 1946).

*Antibacterial activity.* When a colony of *Bacillus polymyxa* on an agar plate was sprayed with a suspension of *Salmonella schottmuelleri* or *Escherichia coli* and compared to a similar plate sprayed with *Staphylococcus aureus*, the difference in the inhibition zones of the gram-negative and the gram-positive organisms was striking, the former showing a wide zone (approximately 40 mm), the latter a relatively narrow zone (approximately 10 mm). It was this difference alone which stimulated further investigation since, at the time the investigation began, no antibiotic had been described which was more active against gram-negative bacteria than gram-positive bacteria.

At first some difficulty was experienced in demonstrating antibacterial activity in bacteria-free broth filtrates. This may have been due to the use of filters which removed the active principle. With the introduction of sintered

<sup>2</sup> The authors wish to thank Miss Nydia H. Ananenko for conducting these two tests in the identification of *Bacillus polymyxa*.

glass filters appreciable activity could be demonstrated. An early antibacterial spectrum obtained with filtered broth is given in table 1.<sup>3</sup>

As shown in table 1, crude fermentation liquor was highly active against the gram-negative bacteria but either inactive or relatively inactive against the gram-positive organisms, confirming and extending the previous findings with the *Bacillus polymyxa* colony. More striking than the results with crude fermentation liquor were those obtained with concentrates of polymyxin. These were relatively free of activity against gram-positive bacteria, even against those organisms, for example, *Diplococcus pneumoniae* SVI, which were some-

TABLE 1  
*Antibacterial spectrum of polymyxin broth filtrates\**

ORGANISM	MEDIUM†	HIGHEST INHIBITORY DILUTION‡
<i>Escherichia coli</i> .....	A, 1/16	1,024
<i>Eberthella typhosa</i> .....	A, 1/16	2,048
<i>Shigella dysenteriae</i> (Flexner).....	A	512
<i>Salmonella schottmuelleri</i> .....	A, 1/16	128
<i>Pseudomonas aeruginosa</i> .....	A, 1/16	128
<i>Klebsiella pneumoniae</i> .....	A, 1/16	512
<i>Streptococcus</i> , group A, strain C203.....	A	8
<i>Streptococcus</i> , group B.....	A, 1/4	4
<i>Streptococcus</i> , group D.....	A, 1/2	0
<i>Diplococcus pneumoniae</i> , type I.....	A	32
<i>Staphylococcus aureus</i> .....	A, 1/16	0
<i>Clostridium welchii</i> .....	B	16
<i>Erysipelothrix rhusiopathiae</i> .....	B+	8

\* The medium consisted of glucose, glycerol, tryptone, yeast extract, and inorganic salts, and was therefore far more complex than the routine production medium which was finally developed (Stansly *et al.*, 1947).

† A = Trypticase-soy-phosphate broth (Baltimore Biol. Lab.). A, 1/2, 1/4, and 1/16, designates the medium used at 1/2, 1/4, and 1/16 the concentration recommended by the manufacturer.

B = Brewer's thioglycolate broth.

B+ = Brewer's thioglycolate broth + bile and yeast extract.

‡ Inhibitory end point obtained by serial twofold broth dilution.

what affected by the crude liquor. Thus from table 1 it can be calculated that *E. coli* is 32 times more sensitive to the broth filtrate than is *D. pneumoniae*. With a partially purified preparation of polymyxin the ratio was found to be in excess of 2,048.<sup>4</sup>

A possible explanation for the difference in behavior of the liquor and concentrates was that the liquor contained at least two active substances, only one of which, the gram-negative principle, was present in the concentrates. In

<sup>3</sup> We wish to thank Mrs. Edith Jackson for conducting the antibacterial spectrum.

<sup>4</sup> We wish to thank Dr. H. J. White and Mrs. A. H. Clapp for the data on the purified preparations.

support of this explanation is the fact that, as described below, it has been possible to extract from the cells of *Bacillus polymyxa* a water-insoluble, ethanol-soluble substance which is highly active against *Staphylococcus aureus* and inactive against *E. coli*. It is suggested, therefore, that the low order of activity of metabolic liquors against gram-positive bacteria may be due to small amounts of this cellular substance escaping into the medium.

Ten grams of moist, unwashed *Bacillus polymyxa* cells and cellular debris, collected by centrifugation, were triturated with sand to a smooth paste. Fifty ml of 95 per cent ethanol were added and the suspension was shaken overnight at room temperature. To 40 ml of the alcoholic filtrate, 80 ml of water were added and the resulting precipitate was collected and dried. It was then dissolved in boiling 95 per cent ethanol and treated several times with charcoal to decolorize it. Water was added to the point of incipient turbidity and the solution cooled. The flocculent white precipitate was washed with ethanol and ether, and dried. A 200-mg per cent suspension was made in water and tested for activity against *E. coli* (MacLeod) and *S. aureus* (Barlow) by the agar streak method. The suspension inhibited *S. aureus* at 10  $\mu$ g per ml and was inactive against *E. coli* at 1,000  $\mu$ g per ml. The origin (Stokes *et al.*, 1942), solubility properties (insoluble in water, ether, chloroform, and acetone), and biological behavior are similar to those of tyrothricin, although its relationship to tyrothricin has not otherwise been determined.

*Effect of blood on activity.* Before therapeutic experiments were instituted it was felt desirable to determine the effect of blood on the antibacterial activity of polymyxin and to determine whether the active substance contained any hemolytic principle. On blood agar plates a colony of *Bacillus polymyxa* showed a very narrow but distinct zone of hemolysis. However, the antibacterial zone (*E. coli*) was much greater.

The experiment summarized in table 2 demonstrated that blood had no appreciable effect on the antibacterial activity of polymyxin, nor had polymyxin, in the concentrations used, any visible effect on blood. Also, the fact that the last tubes showing no growth apparently contained no viable cells suggested that polymyxin had bactericidal properties.

#### *Differentiation of Polymyxin from Known Antibiotics*

Polymyxin is active only against certain gram-negative bacteria (Stansky *et al.*, 1947). This fact alone would distinguish it from all known antibiotics. It may be worth while, however, to point out these and other differences insofar as the literature or actual comparisons in the laboratory permit.

*Tyrothricin.* The insolubility of tyrothricin and its components in water (Hotchkiss and Dubos, 1941; Dubos and Hotchkiss, 1942), its hemolytic activity (Dubos and Hotchkiss, 1942), its toxicity (Robinson and Molitor, 1942), and its greater activity for gram-positive compared to gram-negative organisms (Dubos and Hotchkiss, 1941) distinguished it from polymyxin.

*Streptomycin and streptothricin.* Both streptomycin and streptothricin have gram-positive activity, thus distinguishing them from polymyxin. Neverthe-

less, their similarity to polymyxin in certain other respects was notable. These were their basic nature (Waksman, Bugie, and Schatz, 1944), water solubility (Waksman and Schatz, 1945), high activity against certain gram-negative bacteria (Waksman *et al.*, 1944), similarity in concentration procedure (Waksman and Schatz, 1945; Stansly *et al.*, 1947), and high activity of streptomycin in the *Klebsiella pneumoniae* mouse infection (Heilman, 1945). In view of these similarities, it was felt desirable to compare polymyxin, streptomycin, and streptothricin experimentally to determine whether any close relationships existed among them.

The effect of the pH of the medium on the antibacterial activity of streptomycin and streptothricin is well known (Foster and Woodruff, 1943; Waksman

TABLE 2

*Effect of blood on the antibacterial activity of polymyxin and the effect of polymyxin on blood\**

CONC. POLYMYXIN MG PER CENT	50 PER CENT BLOOD		20 PER CENT BLOOD		10 PER CENT BLOOD		NO BLOOD
	Growth	Hemolysis	Growth	Hemolysis	Growth	Hemolysis	Growth
32	—	—	—	—	—	—	—
1/8	—	—	—	—	—	—	—
1/16	—	—	—	—	—	—	—
1/32	—†	—	—	—	—	—	—
1/64	+	—	—†	—	—†	—	—†
1/128	+	—	+	—	+	—	—
1/256	+	—	+	—	+	—	+

\* Serial twofold dilutions of a crude polymyxin concentrate were made in trypticase-soy-phosphate broth containing the indicated concentrations of defibrinated rabbit blood. Each tube contained a total of 2 ml and was inoculated with approximately 700 *E. coli* cells. Incubation was for 24 hours at 37 C, and the presence or absence of growth was determined by visual inspection. This was possible since the red blood cells had settled by this time.

† These tubes were plated out on agar (1 ml from the tube + 13 ml agar) and incubated for 48 hours at 37 C. No visible colonies appeared on any of the plates.

and Schatz, 1945) and seemed a plausible basis for comparison. Another, obviously, was an antibacterial spectrum with selected organisms. The results of tests using these criteria are shown in tables 3 and 4.

The anticipated increase in activity with increasing pH in the case of streptomycin and streptothricin (table 3) was confirmed, whereas polymyxin showed essentially no change in activity under the same circumstances. The data show that, under the conditions employed, streptomycin was 16 times more active at pH 8.5 than at pH 5.5 and streptothricin 78 times more active at pH 8.5 than at pH 5.5.

The data in table 4 indicate that the preparation of polymyxin used in this experiment was 16 times more active against *E. coli* than was streptomycin, but less than one sixteenth as active as streptomycin against *Bacillus mycoides*. Likewise, the preparation of streptothricin was twice as active as polymyxin against *E. coli* but over 80 times as active against *Bacillus subtilis*. These ob-

servations comprised presumptive evidence for the nonidentity of polymyxin with streptomycin or streptothricin. Cross-resistance experiments with polymyxin and streptomycin confirmed this presumption (White and Clapp, to be published). Additional biological and chemical properties which distinguish polymyxin from streptomycin and streptothricin have been found and will be reported elsewhere.

*Subtilin.* The relative insolubility of subtilin in water at neutrality (anonymous, 1946) and its inactivity against most gram-negative bacteria (Salle and Jann, 1945) distinguished subtilin from polymyxin. The susceptibility of

TABLE 3  
*Effect of pH of assay medium on the inhibition of E. coli*

EXPERIMENT	ANTIBIOTIC†	CONC. IN MG PER CENT INHIBITING GROWTH OF <i>E. COLI</i> * AT INITIAL pH VALUES OF			
		5.5	6.5	7.5	8.5
1	Polymyxin	0.19	0.39	0.39	0.39
	Streptomycin	25.0	25.0	1.56	1.56
2	Polymyxin	0.19	0.09	0.09	0.09
	Streptothricin	1.56	0.39	0.09	0.02

\* In T-S-P medium, agar streak method.

† Antibiotic solutions adjusted to pH 6.4 and titrated in media of indicated pH.

TABLE 4  
*Relative antibacterial activity of polymyxin, streptomycin, and streptothricin*

EXPERIMENT	ANTIBIOTIC	MINIMUM EFFECTIVE CONC.* MG PER CENT		
		<i>E. coli</i>	<i>B. mycoides</i>	<i>B. subtilis</i>
1	Polymyxin	0.5	>32	
	Streptomycin	8.0	2	
2	Polymyxin	0.09		>2,000
	Streptothricin	0.04		25

\* In T-S-P medium, agar streak method.

subtilin to decomposition by pepsin, trypsin, and pancreatin (anonymous, 1946) and the resistance of polymyxin to these enzymes (Stanly and Ananenko, to be published) confirmed the lack of identity.

*Bacitracin* (Johnson, Anker, and Meleney, 1945). Its activity against gram-positive bacteria and lack of activity against gram-negative bacteria were the only criteria available which served to distinguish bacitracin from polymyxin.

*Eumycin* (Johnson and Burdon, 1946). The solubility of eumycin in acetone and its inactivity against *Eberthella typhosa* and *E. coli* distinguished it from polymyxin.

*Gramicidin S.* Its insolubility in water (Belozersky and Passhina, 1944),



toxicity (Gause and Brazhnikova, 1944), hemolytic activity (Manevich and Pitskhelauri, 1944), and greater or equivalent activity against gram-positive organisms compared to gram-negative organisms (Gause and Brazhnikova, 1944) distinguished this substance from polymyxin.

*Colistatin* (Gause, 1946). Its higher activity against staphylococci than against *E. coli* and its inextractability from broth filtrates with normal butanol were characteristics distinguishing this recently described material from polymyxin.

*Bacillin* (Foster and Woodruff, 1945). Bacillin is equally effective against gram-positive and gram-negative bacteria. Blood neutralizes its activity *in vitro*. These facts distinguished bacillin from polymyxin.

*Antibiotic from Bacillus licheniformis* (Callow and Hart, 1946). Its greater activity against *S. aureus* than *E. coli*, activity against *Mycobacterium tuberculosis*, and apparent insolubility in ethanol distinguished this recently described material from polymyxin.

#### SUMMARY

The isolation and identification of *Bacillus polymyxa* as the organism producing the antibiotic polymyxin is described. Preliminary data on the biological activity of polymyxin which served both to distinguish and characterize the antibiotic are given. The points of distinction between polymyxin and some known antibiotics which bore a superficial resemblance to polymyxin are discussed.

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# MICROBIOLOGICAL AGENCIES IN THE DEGRADATION OF STEROIDS

## II. STEROID UTILIZATION BY THE MICROFLORA OF SOILS

G. E. TURFITT

*Home Office Forensic Science Laboratory, Nottingham, England*

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An investigation of the degradation of cholesterol by soil microorganisms has shown that the initial oxidation of this compound is due, both *in situ* and in pure culture, to the activities of members of a single genus, *Proactinomyces* (Turfitt, 1944a). During the cultural work a considerable number of organisms, both molds and bacteria, persisted with the strains of *Proactinomyces* through many subcultures, but when isolated and inoculated into synthetic medium with cholesterol as sole C source, were unable to survive. Since *Proactinomyces* oxidation results in the formation of  $\Delta^4$ -cholestenone, and subsequently in actual molecular fission (Turfitt, 1944b, 1947), it may well be that the further products resulting from *Proactinomyces* oxidation constituted a substrate for the growth of the attendant organisms.

In the case of steroid compounds lacking the intact hydrocarbon C<sub>17</sub> side chain, oxidations at —OH groups giving the corresponding keto compounds have been reported with several groups of organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1942), *Flavobacterium androstenedionicum* (Ercoli and Molina, 1944), *Flavobacterium carbonilicum* (Molina and Ercoli, 1944), *Alcaligenes faecalis* (Schmidt, Hughes, Green, and Cooper, 1942; Hughes and Schmidt, 1942), *Escherichia coli* (Schmidt and Hughes, 1944), and pseudodiphtheria bacilli (Zimmermann and May, 1944). These investigations, however, have been concerned essentially with ketone formation, and, in general, alternative carbon sources such as serum or yeast water have been included in the cultures with the object of obtaining a vigorous multiplication of the bacterial cells and a consequent high dehydrogenase concentration. Synthetic media with cholic acid as the sole carbon source have been used by Schmidt, Hughes, Green, and Cooper (1942) in oxidations with *Alcaligenes faecalis*, and triketocholanic acid has been isolated as the end product of the bacterial action. In this instance it is clear that the carbon available for growth has been derived from a breakdown of the bile acid molecule.

The only general restrictive influence on bacterial utilization thus far reported is due to the side chain, and the available evidence suggests that the modified natural sterol skeleton is probably susceptible to attack by a variety of organisms.

### EXPERIMENTAL RESULTS

Although the basic ring structure of all steroid compounds is fundamentally the same, the variety of compounds resulting from the introduction of various

substituents is extremely wide and is in addition vastly increased by the complex stereoisomeric configurations in the steroid rings. It was clearly impracticable to investigate more than a limited selection of compounds, and a choice was made with a view to determining the influence on the microbiological utilization of (1) the length and nature of the side chain, (2) the presence of free and esterified —OH groups, (3) the presence of ketonic groups in the more usual C<sub>3</sub> and C<sub>17</sub> positions, (4) the presence of free or "blocked" double bonds, (5) the *cis*- and *trans*-decalin configurations of rings A and B, (6) stereoisomerism at C<sub>5</sub>, and (7) the presence of benzenoid rings in place of the complete saturation of the polyhydrocyclopentanophenanthrene skeleton.

*Isolation technique.* The process of selective isolation used in the investigation of the cholesterol-decomposing organisms of soils (Turfitt, 1944a) was again adopted, although certain modifications were considered desirable owing to the varied physical and chemical properties of the steroid substrates.

The soil samples were restricted to the British Isles, and the 20 specimens taken constituted a range covering both virgin and cultivated ground. In each instance the sample was collected from immediately below the soil surface in a sterile 4- by 1-inch tube. No attempt was made to investigate the microflora present at a greater depth.

Sterilization of the individual steroid materials to be used in the cultures presented a somewhat difficult problem. In the case of cholesterol, steam sterilization was entirely effective, but this method was obviously unsuitable with other steroids.  $\Delta^4$ -Cholestenone, for example, has a melting point of 80 C and on cooling separates as a hard, solid mass. Again, there can be no guarantee that in the case of some of the less stable steroids heat treatment does not result in a slight decomposition yielding a material containing traces of impurity. The same considerations hold with regard to sterilization by ultraviolet light, since with ergosterol, and probably also in minor degree with certain other steroids, molecular transformation results. A successful solution of the problem was achieved by recrystallization of the compounds from suitable solvents, with filtration and drying conducted under aseptic conditions. The materials were stored in sterile tubes and were tested for sterility before use by streaking on both nutrient agar and wort agar plates.

*Bacteria and actinomycetes.* Conical culture flasks (100-ml), each containing 30 ml mineral salt solution (NH<sub>4</sub>NO<sub>3</sub>, 0.1 per cent; K<sub>2</sub>HPO<sub>4</sub>, 0.025 per cent; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.025 per cent; NaCl, 0.0005 per cent; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.00001 per cent) were autoclaved at 115 C for 10 minutes, and approximately 1 mg steroid was introduced aseptically.

*Molds.* In devising a method to ensure a normal surface development of mold mycelium, a distinction was drawn between the slightly soluble carboxylic acids, cholic acid, and 3-hydroxy- $\Delta^5$ -cholenic acid, and the insoluble steroid compounds. For the former, 100-ml Gates' culture flasks, each containing 50 ml of mineral salt solution (NaNO<sub>3</sub>, 0.2 per cent; KH<sub>2</sub>PO<sub>4</sub>, 0.1 per cent; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05 per cent; KCl, 0.05 per cent; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 per cent), were sterilized by steaming for 1 hour on each of 3 successive days, and ap-

proximately 1 mg steroid was aseptically added. In the case of the insoluble compounds petri dishes were employed; each contained a thick, compact layer of glass wool previously purified by acid and alcohol treatment and just covered with the mineral salt solution. The dishes were sterilized by steaming, and approximately 1 mg steroid was sprinkled with aseptic precautions over the surface.

Approximately 0.5 ml of a heavy aqueous suspension of each of the 20 soil samples were transferred to each of the flasks and dishes containing the various steroid compounds, and the cultures were incubated aerobically at 25 C. After 7 days a loopful from each vessel was transferred to a duplicate containing fresh medium, this procedure being repeated three times. From the final cultures transfers were made on (a) nutrient agar, (b) casein agar, and (c) Czapek-Dox agar. The organisms which appeared on these plates were isolated and incubated in pure culture with the various steroid-containing media. Increased bacterial count or development of mold mycelium, together with alteration of the pH of the medium, was regarded as evidence of steroid utilization.

*Description and distribution of isolated organisms.* In this investigation of the aerobic organisms of soils, 20 soil samples and 20 steroid compounds, under two separate cultural conditions, involved 800 initial cultures. After the subsequent "purification" cultures, numbers of organisms were isolated which failed to survive on the appropriate pure steroid. No mention is made of these organisms in table 1, which summarizes the numbers and general types of steroid-decomposing organisms isolated from particular classes of soils. Strains of *Proactinomyces* are indicated by "P," and the numbers of strains isolated are given in parentheses. Gram-negative rods are indicated by "gm-."

*Description of isolated strains.* In this survey, 313 of the 355 cultures of bacteria isolated consisted of gram-positive rods, or of long or short filaments breaking up in older culture into short rods or coccoid forms. The organisms have been cultured on a wide variety of media, and in the majority of cultures, especially upon the less rich media, aerial mycelium was produced in greater or less degree; in no instance was there evidence of spore formation upon examination by the method of Orskov (1923). In cultural and morphological characteristics the organisms fall essentially within the genus *Proactinomyces* and for the most part have the softness and translucency of the  $\alpha$ -type of colony (Umbreit, 1939). In several cases the strains did not show a strict agreement with the characteristics of known types, but the divergencies were insufficient to justify new species rank, and they have been regarded rather as variants of existing species. The divergencies were particularly marked in respect to acid-fastness, a character which was found to be influenced markedly by the composition of the culture medium. This feature of *Proactinomyces* has previously been reported by Jensen (1931, 1932) in a detailed taxonomic study of the genus.

The 298 cultures of this group have thus been classified as follows: *P. opacus* (135 cultures), *P. erythropolis* (126 cultures), *P. globerulus* (17 cultures), *P. coeliacus* (8 cultures), *P. aquosus* (5 cultures), *P. crystallophagus* (5 cultures), and *P. agrestis* (2 cultures).

TABLE 1  
*Steroid-decomposing organisms from varied soil types*

STEROID	SOIL TYPES (4 SAMPLES EACH TYPE)				
	Acid Sand	Loam	Marl	Alkaline Peat	Arable
Stigmasterol	P (3)	P (4)	P (4)	P (2)	P (6)
$\beta$ -Sitosterol	P (4)	P (4)	P (5)	P (3)	P (4)
Ergosterol	P (3)	P (5)	P (3)	P (3) gm (1)	P (6) gm (2)
Coprosterol	P (3)	P (3)	P (3)	P (2)	P (5)
Dihydrocholesterol	P (4)	P (4)	P (4)	P (3)	P (7)
<i>epi</i> -Dihydrocholesterol	P (3)	P (3)	P (4)	P (3)	P (6)
Cholesterol acetate	P (3)	P (4)	P (3)	P (2)	P (5)
Coprosterol acetate	P (3)	P (4)	P (4)	P (3)	P (6)
Cholesteryl chloride	None	None	None	None	None
Cholesterol acetate di-bromide	None	None	None	None	None
Dicholesteryl ether	P (4)	P (4)	P (4)	P (2)	P (4)
$\Delta^4$ -Cholestenone	P (4)	P (5)	P (4)	P (3)	P (6)
Coprostanone	P (3)	P (3)	P (4)	P (3)	P (5)
Androsterone	P (4) gm (2)	P (5) gm (1)	P (5)	P (3)	P (6) gm (1)
<i>trans</i> -Dehydro-androsterone	P (4) gm (3)	P (4) gm (2)	P (4) gm (1)	P (2) gm (1)	P (6) gm (3)
Progesterone	P (3) gm (1)	P (3) gm (1)	P (3) gm (1)	P (3)	P (4) gm (2)
3-Hydroxy- $\Delta^4$ -cholenic acid	P (4) gm (3) molds (1)	P (5) gm (2)	P (4) gm (1)	P (3) gm (1)	P (5) gm (2) molds (1)
Cholic acid	P (4) gm (5) molds (2)	P (4) gm (3) molds (1)	P (3) gm (1)	P (3)	P (4) gm (2) molds (1)
$\alpha$ -Oestradiol	P (1)	None	None	None	P (2)
Oestrone	None	None	None	None	P (1)

Many of these individual cultures, of course, originating from the same soil samples, were quite obviously identical. In addition, two cultures have been provisionally identified as *Mycobacterium phlei*. An authentic strain of *M. phlei* has previously (Turfitt, 1944a) been found unable to utilize cholesterol, and in consequence tests for cholesterol-decomposing ability have been conducted with a number of standard strains of this organism. The property, which was fairly vigorous in some strains, was entirely lacking in the majority and could not, furthermore, be stimulated by enrichment cultures. Closely comparable findings have been experienced with cultures of *Mycobacterium smegmatis* and *Mycobacterium stercoreis*.

The  $\beta$ -group of *Proactinomyces* was represented by 13 cultures which have been thus identified: *P. asteroides* (8 strains), *P. farcinicus* (4 strains), and *P. paraffinae* (1 strain).

In addition to the *Proactinomyces*, 42 cultures of gram-negative bacteria were obtained. Of these, 29 were short rods with 1 to 5 polar flagella and on asparagine agar (Georgia and Poe, 1931) developed the green fluorescence of *Pseudomonas*. The blue pigment pyocyanin, typical of *Pseudomonas aeruginosa*, was not detected even in glycerol peptone agar (Gessard, 1891; Turfitt, 1936). Neither these nor the 13 cultures of gram-negative nonfluorescent organisms have as yet been satisfactorily classified, but they are being incorporated in a further study of steroid utilization specifically by gram-negative organisms. Ercoli (1938), in the attempted bacterial reduction of male sex hormones to etiocholane derivations, has described the culture of *Pseudomonas fluorescens*, and also of *Escherichia coli*, in meat broth in the presence of 200 mg androstenedione. No hydrogenation products could be isolated, but 86 mg of unchanged dione were recovered. Similar results were obtained with *trans*-dehydroandrosterone. This apparent utilization of the steroid by these organisms is thus in accord with the present results indicating that in such modified steroids gram-negative bacteria play a not inappreciable part.

The only instances in which isolated molds were able to survive repeated transfer in pure culture were with the free acids, cholic acid, and 3-hydroxy- $\Delta^5$ -cholonic acid. In all, six molds (identified as species of *Penicillium*, *Aspergillus*, and *Alternaria*) were found to yield a few straggling hyphae on the surface of the medium with the petri dish, glass wool technique. A definite mycelial felt never developed. There was no change in the pH of the medium, and no detectable ketone formation. Steroid decomposition by these organisms was clearly of a negligible order, and they were accordingly not further investigated.

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#### SUMMARY AND CONCLUSIONS

Cultural conditions are described by which the microflora of soils have been tested for ability to utilize a variety of steroid materials.

Steroids generally, with a very few special exceptions such as halogen-substituted derivatives, are attacked by *Proactinomyces* of soils, and these are clearly the predominant organisms in steroid decomposition.

With steroid compounds in which the C<sub>17</sub> side chain is modified or lacking, certain gram-negative bacteria, especially of the fluorescent type, can utilize the molecule.

Although species of *Penicillium*, *Aspergillus*, and *Altenaria* have been found to survive repeated transfer in pure culture on soluble carboxylic acid derivatives, the paucity of the growth and the lack of evidence of steroid decomposition are taken to indicate that these fungi are of small significance in the utilization of steroids in nature.

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# PLATE METHODS FOR TESTING ANTIBIOTIC ACTIVITY OF ACTINOMYCETES AGAINST VIRULENT HUMAN TYPE TUBERCLE BACILLI<sup>1</sup>

ELIZABETH H. WILLISTON, PARI ZIA-WALRATH, AND GUY P. YOUMANS  
*Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois*

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A simple way to test the antibiotic properties of an organism is to streak it on an agar plate and then, after growth has been established, cross-streak with the organism against which it is to be tested (Waksman, 1945). The zone of inhibition of the test organism can then be measured. Thus, by cross-streaking with many organisms, it becomes fairly easy to establish a "spectrum" of the inhibiting properties of any bacterium, mold, or actinomycete which will grow discretely on an agar plate.

Once the bacteriostatic properties of an agent have been established, there are many ways of testing it quantitatively. Extracts and filtrates of the culture of the effective organism may be tested by serial broth dilutions, turbidimetric measurements, agar plate dilutions, and cylinder plate methods (Waksman, 1945). Animal tests may supplement these methods after a nontoxic extract or filtrate has been prepared.

Serial dilution methods and animal tests have been useful in measuring the reaction of antibiotic extracts on virulent human type tubercle bacilli. Bush, Dickinson, Ward, and Avery (1945) report the use of the cylinder plate method with the rapidly growing nonpathogenic strain of tubercle bacillus known as "607," but slowness of growth and difficulties in preparing suspensions of virulent human type tubercle bacilli are probably responsible for the fact that the cross-streak and the cylinder plate method have not, to the knowledge of the authors, been reported using these organisms.

This paper reports three agar plate methods which have been used to select actinomycetes with antibiotic properties and to test quantitatively filtrates and concentrates derived from these.

## METHODS AND RESULTS

*Cross-streak method.* Thirteen strains of actinomycetes<sup>2</sup> were selected for the tests. Seven media were chosen which would promote the growth of the actinomycetes and to each of these were added glycerol to the amount of 2 per cent and agar to the amount of 1.5 per cent. The glycerol may be omitted but the growth of tubercle bacilli is slower. The complete formulae for these media follow:<sup>3</sup>

<sup>1</sup> This work was aided by a grant from Parke, Davis and Company, Detroit 32, Michigan.

<sup>2</sup> Obtained from Dr. John Ehrlich of Parke, Davis and Company, Detroit, Michigan.

<sup>3</sup> Formulae for these media were furnished by Dr. John Ehrlich, Parke, Davis and Company, Detroit, Michigan.

*Medium 1*

Corn steep liquor (Corn Prod. Ref.).....	1.0%
K <sub>2</sub> HPO <sub>4</sub> .....	0.2%
NaCl.....	0.5%
Cerelose (Corn Prod. Ref.).....	1.0%

*Medium 2*

Corn steep liquor.....	1.0%
K <sub>2</sub> HPO <sub>4</sub> .....	0.2%
NaCl.....	0.5%
Maltose, tech. (Difco).....	1.0%

*Medium 3*

Curbay B-G (U. S. Indus. Chem.).....	0.5%
Casamino acids (Difco).....	0.5%
NaCl.....	0.5%
Cerelose.....	1.0%

*Medium 4*

B-Y fermentation solubles (Comm. Solv. Corp.).....	0.5%
Casamino acids.....	0.5%
NaCl.....	0.5%
Cerelose.....	1.0%

*Medium 5*

Beef extract (Difco).....	0.3%
Peptone (Difco).....	0.5%
Maltose.....	1.0%

*Medium 6*

Corn steep liquor.....	1.0%
K <sub>2</sub> HPO <sub>4</sub> .....	0.2%
NaCl.....	0.5%
Maltose cp.....	1.0%

*Medium 7*

Beef extract.....	0.3%
Peptone (Difco).....	0.5%
NaCl.....	0.5%
Glucose.....	1.0%

These media were adjusted to a pH of 7.0, tubed in 40-ml amounts, and stored in the icebox until needed. When melted and poured into plates this amount of medium helped to provide for loss by evaporation. The actinomycetes were streaked on the agar plates with a 4-mm loop from a spore suspension made by pouring saline over a sporulating slant and loosening the spores with a loop, or directly from a more stable preparation made by mixing the spores in a gelatin suspension and drying. These plates were incubated at 24 C for 5 days, or until a streak of growth about 1 cm in width had been established.

A thick suspension of the H37Rv strain of *Mycobacterium tuberculosis* was obtained by grinding a 14- to 21-day-old pellicle growth from a flask of Proskauer

and Beck synthetic medium and diluting with 0.01 molar phosphate buffer solution until a thick, pasty preparation was obtained. This thick, pasty inoculum was necessary to give uniform streak growth.

Streaks of H37Rv were made with a 4-mm loop at right angles to the actinomycete streak, and the plates incubated at 37 C. The growth of tubercle bacilli was at a maximum in 2 to 3 weeks and appeared as a wide rugose band 2 or 3 times the width of the original inoculating loop. The degree of inhibition of the tubercle bacillus was measured in millimeters from the edge of the actinomycete streak. Where several streaks of the same strain of tubercle bacillus were made, the readings were averaged. Several plates were also streaked with both H37Rv and H37RvR, the latter a strain of H37Rv which had been made resistant *in vitro* to more than 1,000 micrograms of streptomycin per ml of medium (Williston and Youmans, 1947). Figure 1, nos. 1, 2, and 3, show results of cross-streaking actinomycete plate cultures with H37Rv and H37RvR.

At the time the results were observed, the hydrogen ion concentration of the agar adjacent to the streak was determined in order to eliminate inhibition due to acidity alone. The hydrogen ion concentration was determined by cutting out strips of the agar and dissolving them in distilled water in the cup of a Coleman electrometer. Table 1 shows the width of the zone of inhibition (in mm) of the H37Rv strain of tubercle bacillus by 14 strains of actinomycetes on seven different media chosen because they favored growth and antibiotic production by the actinomycetes. Table 2 shows the results obtained on two media comparing the degree of inhibition of growth produced by the actinomycetes on the virulent H37Rv and the avirulent 607 strain. Not only are the organisms inhibited to a different degree, but several actinomycetes inhibited the virulent H37Rv and not the avirulent 607 strain. Obviously, if only the avirulent strain were used in these tests, effective antibiotics might be missed.

*Cylinder plate method.* This method was used in an attempt to make quantitative studies on filtrates and extracts of cultures which had already shown inhibitory properties. The medium used was a modified Proskauer and Beck synthetic medium to which was added 1.5 per cent agar. Forty ml of the nutrient agar were first put in the plate and allowed to harden, and then a 4-ml quantity of the agar that had been seeded with 7.5 mg (Hopkins tube) of tubercle bacilli per ml of agar was poured over the surface. This inoculum of tubercle bacilli for the seeded layer was ground with mortar and pestle until very smooth so that the opacity of the growth layer was uniform after incubation. Stainless steel cylinders were dropped gently through a plastic "guide" onto the plates. Into these cylinders were delivered the diluted extracts or filtrates. The plates were incubated 2 or 3 weeks and the diameters of the zones of inhibition were measured in mm. Similar pour plates were also made using the streptomycin-resistant strain of H37RvR. The cylinders were refilled when necessary, from time to time, with the extracts or filtrates to replace loss of potency due to exposure at incubator temperature.

Cylinder plates were made using four cylinders to a plate. Two of the cylinders on each plate contained 10 and 5 micrograms, respectively, of strep-

tomycin per ml. These consistently gave zones of inhibition of tubercle bacilli of approximately 25 and 15 mm, respectively, and served as controls. Filtrates and concentrates of the antibiotics to be tested were placed in two different

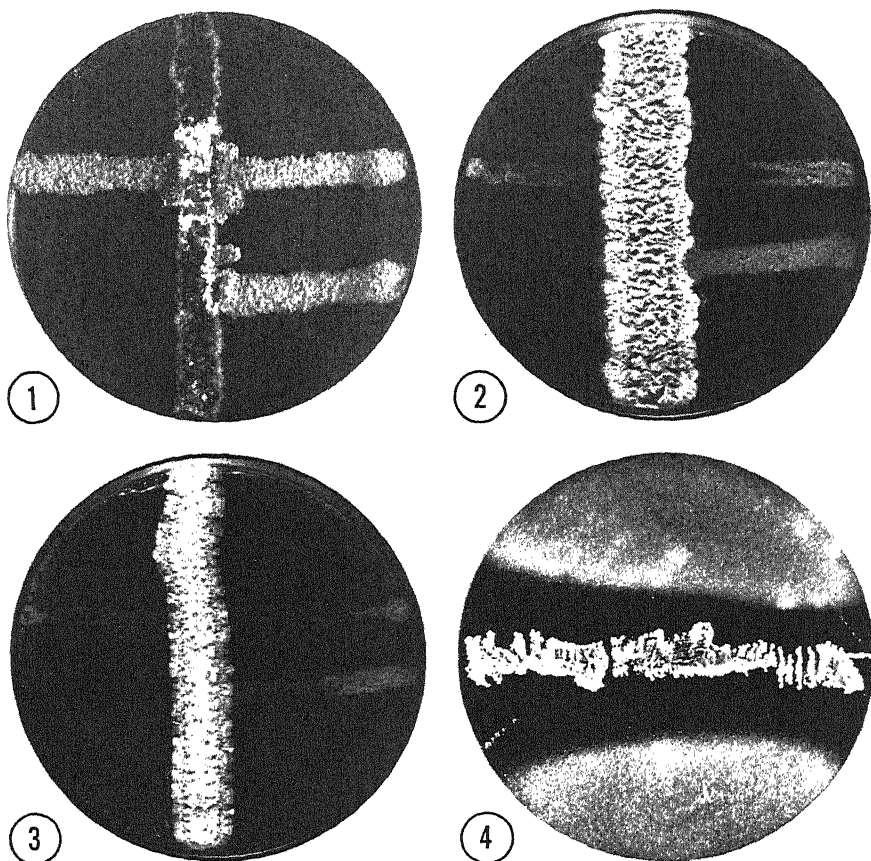


FIG. 1. ACTINOMYCETE CROSS-STREAKED WITH TUBERCLE BACILLUS

Vertical streak: Actinomycete. Upper horizontal streak: H37Rv. Lower horizontal streak: H37RvR (resistant to >1,000 micrograms streptomycin).

No. 1. No inhibition of either streptomycin-sensitive or streptomycin-resistant tubercle bacilli.

No. 2. Inhibition of streptomycin-sensitive strain only.

No. 3. Inhibition of both streptomycin-sensitive and streptomycin-resistant strains.

No. 4. "Streak pour plate" seeded with H37Rv and cross-streaked with an inhibitory organism.

dilutions in the other cylinders. A comparison with streptomycin could thus be established.

Since the margins of the zones of inhibition were usually very fuzzy and indistinct, quantitative data were difficult to obtain. In some cases, however, clear-cut zones were noted.

*Streak plates seeded with tubercle bacilli.* The plates seeded with tubercle

TABLE 1

*Plate cultures of actinomycetes cross-streaked with virulent human type tubercle bacilli H37Rv and H37RvR\**

ACTINOMYCETE CULTURE NO.	AMOUNT OF INHIBITION IN MILLIMETERS							
	Medium							
	1	2		3	4	5	6	7
	H37Rv	H37Rv	H37RvR*	H37Rv	H37Rv	H37Rv	H37Rv	H37Rv
1	>37	25	25	6	3	13	>32	
2	17	15	18	0	0	1	21	
3	20	0	0	6	7	1.5	17	
4	27	9.2	0	11	10	11	27	
5	14	11.3	6.3	11.3	0	7	15	
6	20	16	17.5	11	0	10	17	20
7	13	2.5	4	0	0	2	14	
8	0	0	0	0	0	23	0	0
9	17	19.6	0	8	10	15	15	
10	16	18	15	0	26	3	18	
11	0	0	0	0	0	0	0	0
12	20	3	4	10.6	11	8.3	12	
13	15	15	20	12.5	11.6	18	14	15
<i>S. griseus</i> †	20	12	0			12	20	0

\* Resistant to streptomycin.

† Furnished through the courtesy of Dr. Selman A. Waksman, New Brunswick, New Jersey.

TABLE 2

*Comparison of streak test results obtained with H37Rv and 607*

ACTINOMYCETE CULTURE NO.	AMOUNT OF INHIBITION IN MILLIMETERS			
	Medium 6		Medium 1	
	H37Rv	607	H37Rv	607
1	>32	7	>37	13
2	21.3	16	17.1	11.2
3	17.3	16	20.2	10.7
4	27	11.2	27	13.5
5	15	6	14	6.5
6	17	17	20	13
7	14	0	13	0
8	9	0	0	0
9	15	20	17	20.7
10	18.2	0	15.8	0
11	0	0	0	0
12	12	0	20	0
13	20.4	18.5	19.2	15
<i>S. griseus</i>	20.2	21.5	20.5	?

bacilli prepared as described above were also used for streaking the actinomycete cultures. These were incubated first at 24 C for 5 days, then at 37 C for 2 weeks.

If any of the actinomycetes possessed bacteriostatic properties, a zone of inhibition of the tubercle bacilli growing in the agar appeared next to the streak.

Pour plates were seeded with both the resistant H37Rv and the sensitive strain. Eight of the actinomycetes were cross-streaked and the inhibition zones measured. These inhibition zones were approximately the same as those obtained by cross-streaking the actinomycete with tubercle bacilli, as recorded in table 2. Figure 1, no. 4, shows an inhibitory organism cross-streaked on a pour plate seeded with H37Rv.

#### CONCLUSIONS

The streak plate method using the virulent type of tubercle bacillus (H37Rv) is useful for the testing of the antibiotic properties of actinomycetes. This gives a relatively rapid method for screening cultures in a search for new antibiotics. If a streptomycin-resistant strain of H37Rv is also streaked on the plates, cultures bearing a relationship to *Streptomyces griseus* may be detected.

A smooth, opaque layer of growth may be obtained by seeding pour plates with H37Rv. Filtrates and concentrates in cups will give inhibition zones, though quantitative measurements are difficult to make because the zones are not always sharply defined.

Pour plates, seeded with tubercle bacilli and streaked with actinomycetes, are useful in the search for cultures with tuberculostatic properties. The plates may be seeded with H37Rv or with H37RvR (resistant to streptomycin) and cross-streaked with various strains of actinomycetes.

The avirulent, rapidly growing strain 607 is not suitable for this purpose, since some strains of actinomycetes which inhibit the virulent H37Rv strain do not inhibit, under the same conditions, strain 607.

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# A MORPHOLOGICAL VARIANT OF *ESCHERICHIA COLI* AND ITS RESISTANCE TO STREPTOMYCIN

ESTHER STUBBLEFIELD

*The Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

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During a study of acquired resistance of bacteria to streptomycin a culture of *Escherichia coli* was found to give rise to a round-cell variant which could be maintained in culture. Although the effect of streptomycin on the original occurrence of these cells could not be definitely established, a study of the organism and the effect of streptomycin on it has been made. Round-cell forms of *Escherichia coli* have been reported by many workers as occurring in old cultures, in cultures under slightly toxic influences, and in cultures recently isolated from natural sources. In only a few instances have the round cells been found to develop, as such, unmixed with rods.

Coccoid cells very similar to those encountered here have been reported by Hussong (1933). He obtained a culture containing only round cells when a strain of *Escherichia coli*, which had been carried in lithium chloride broth, was transferred to lithium-chloride-free medium. The round cells returned to the rod form when grown again in a medium containing lithium chloride, but several transfers in lithium-chloride-free medium could be made before rods appeared in the culture. Mellon (1925a, 1925b) found round cells in a culture of *Escherichia coli* obtained from the urine of a patient who had been receiving urotropin and sodium acid phosphate. He was able to produce the same forms when a normal culture of *Escherichia coli* was grown in broth containing disodium glycerophosphate and sodium chloride. When this culture was transferred to a plain agar slant, only the round cells developed. On additional incubation these sprouted into coarse filaments and rods.

Dienes (1939, 1942, 1946) studied extensively the "large bodies" of many varieties of bacteria and their relation to the L type of colony. His studies on *Escherichia coli* were made with cultures from pathological urine specimens. He observed the germination of the large forms of *Escherichia coli* into both pleuropneumonia-like and bacterial colonies. He states that in his experience the large bodies produced by toxic influences never germinate and reproduce. The naturally occurring round forms he studied were fragile, difficult to stain, and difficult to transfer. This is not the case with the cells reported here.

Altire-Werber *et al.* (1945) reported bipolar rounded bodies which occurred in the urine of patients treated with penicillin. These reverted to typical *Escherichia coli* on culture. Price *et al.* (1947) reported similar forms in cultures of *Eberthella typhosa* cultivated in broth containing streptomycin.

## EXPERIMENTAL RESULTS

The culture of *Escherichia coli* used in this study had been carried in stock 11 years. The procedure for inducing resistance was to streak a series of meat

infusion agar plates containing streptomycin with an aqueous suspension of the organisms, incubate the plates 48 hours, select a colony from the plate containing the highest concentration of streptomycin, transfer it to a plain meat infusion agar slant, and after 24 hours' incubation repeat the process. It was on the sixth exposure to streptomycin that a colony, picked from a plate containing 100 micrograms of streptomycin per milliliter of agar, was found on transfer to plain agar to grow entirely as globular cells. Only the one colony was picked from this plate; later the culture from which this variant had come was restreaked and 8 colonies were picked and included in the resistance study. A total of 68 colonies were examined at various concentrations of streptomycin. None of these colony transfers showed a difference in morphology from the typical rod form of the parent culture. Another experiment was started with the parent culture in which 20 colonies were selected from each plate containing streptomycin and control colonies were selected from meat infusion agar. Four hundred and forty-three transfers from colonies growing on agar containing various concentrations of streptomycin and 449 transfers from colonies growing on plain agar were examined. All were rod forms. The variant form apparently was selected only by chance.

The atypical culture when growing directly on agar containing streptomycin was a mixture of bizarre rods varying in size, some curved, branching, and Y forms, and a few round forms. A colony consisting of these cells when transferred to a plain agar slant grew only as round cells. These cells varied in size from about 1 to 7 microns and occurred singly, in pairs, chains, and groups; occasionally they gave the appearance of small cells budding from the larger ones. There was some variation in the density with which the different cells stained. All forms were gram-negative. The appearance of a large capsule surrounding the cells was characteristic of all stained preparations of the round cells. Figures 1 and 2 are photomicrographs of the two morphological forms.<sup>1</sup>

The colonies of the variant on agar containing streptomycin were small, smooth, and nonmucoid; colonies of the round cells on plain agar were smaller than the parent *Escherichia coli* and were mucoid but not spreading. The mucoid character of the round-cell culture was most apparent on an agar slant.

Studies were made of the cultural characteristics of the round-cell culture, of the parent culture, of a rod form of *Escherichia coli* which had become resistant to streptomycin, and of a culture of the variant after it had entirely reverted to the rod form. Reactions in nine sugars (glucose, maltose, lactose, sucrose, sorbitol, mannitol, salicin, raffinose, and xylose); tests for the production of nitrites, indole, and hydrogen sulfide; the liquefaction of gelatin; and the reaction in milk were the same for all cultures and were characteristic for *Escherichia coli*. The only differences noted were an occasional failure of the round form to grow in a few media and delayed growth of the variant in all media. After 24 hours' incubation gas production by the round-cell cultures lagged considerably behind that by the rod cultures; at 48 hours it was equal.

Antigenic studies were made on cultures of the parent strain, on the round

<sup>1</sup> The photomicrographs were made by Mr. Norman Drake.



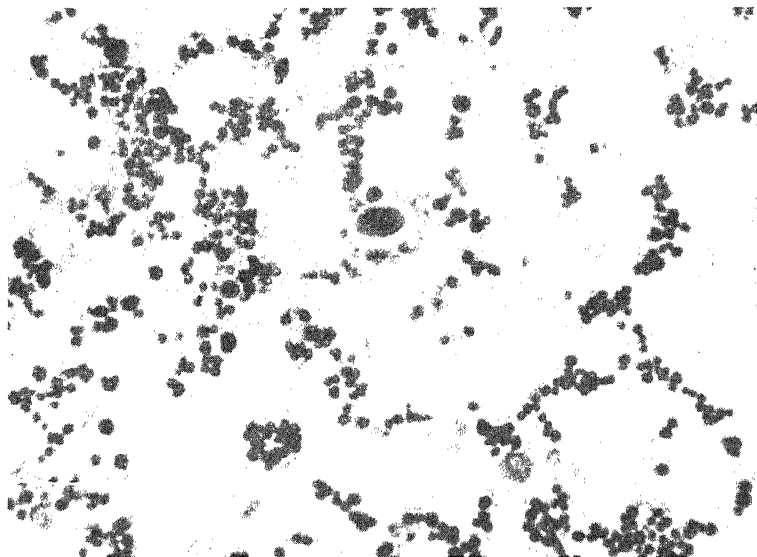


FIG. 1. ROUND VARIANT OF *ESCHERICHIA COLI*, 24-HOUR GROWTH ON PLAIN AGAR. CA. 1,000  $\times$

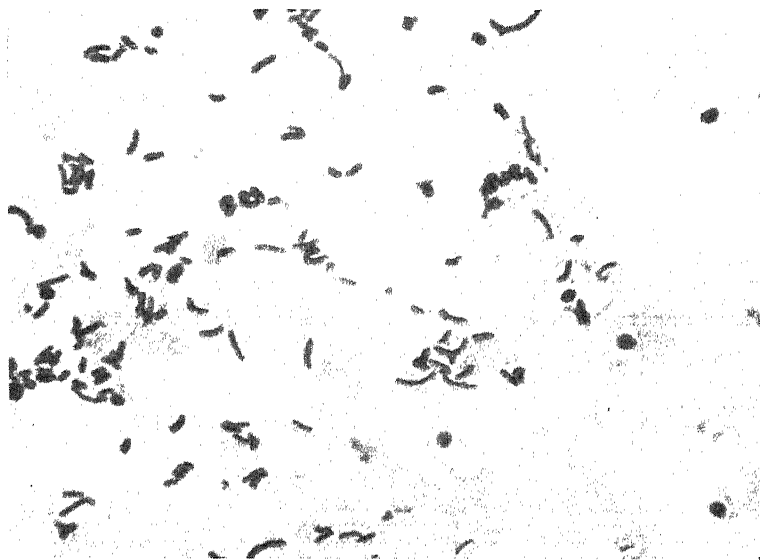


FIG. 2. SAME VARIANT OF *ESCHERICHIA COLI*, FROM A 48-HOUR COLONY ON AGAR CONTAINING STREPTOMYCIN. CA. 1,000  $\times$

form, which at that time was resistant to 10,000  $\mu$ g streptomycin per ml, and on a rod form resistant to this same concentration of streptomycin. All antigens were agglutinated to titer with antisera against both the parent and resistant

rods. Antiserum against the round cells gave a titer of 1:1,600 with the homologous antigen but only 1:400 with the heterologous antigens. These cross-reaction studies demonstrated an antigenic similarity of the two rod forms but an antigenic difference in the round and rod forms. Adsorption studies confirmed the similarity of the two rod forms and demonstrated the presence of an antigen in the round cells which was not present in the rods.

The atypical round form was maintained by alternate transfers on agar containing streptomycin and plain agar. It has been carried through 67 exposures to streptomycin. For the first 22 exposures the plain agar slant transfer was found to contain only the globular cells. From the twenty-second to the sixty-seventh exposure plump rods were seen rarely in some of the agar slant cultures.

A round-cell culture (20 exposures to streptomycin; resistant to 500  $\mu\text{g}$  per ml) was transferred daily on plain agar and in plain broth. For the first 6 agar transfers and for 2 broth transfers the cultures were all round cells. After 14 transfers on agar or after 7 in broth the round forms had entirely disappeared and the cultures contained only small rods. For an additional 16 transfers no round cells were seen. Another round-cell culture much later in the series (51 exposures to streptomycin; resistant to 10,000  $\mu\text{g}$  per ml) required 23 transfers on plain agar before a preparation containing only rod forms was seen, and as this transfer series was continued it was found that a few round cells recurred.

The fact that the variant form became resistant to streptomycin was thought to be of particular interest. However, the total number of exposures necessary before the round organism became resistant to 10,000  $\mu\text{g}$  of streptomycin per ml agar was much greater than the number required for 11 other species of gram-negative rods and the 8 colony subcultures of *Escherichia coli* obtained by the same method of random selection of one colony per plate. The parent strain of *Escherichia coli* was resistant to only 10  $\mu\text{g}$  streptomycin per ml. At the sixth exposure, when the variant appeared, the rod had become resistant to 100  $\mu\text{g}$  per ml. The variant continued to increase in resistance; on the eighth exposure it was resistant to 250  $\mu\text{g}$  per ml and on the tenth to 500  $\mu\text{g}$  per ml. Its resistance did not increase again until the thirty-fourth exposure, when it grew on 2,000  $\mu\text{g}$  per ml, and on the next exposure, on 10,000  $\mu\text{g}$  per ml. The round-cell culture was then grown alternately on agar containing 10,000  $\mu\text{g}$  streptomycin per ml and on plain agar for 38 more exposures; no higher concentrations were tested. With this continued culturing at the same high concentration the growth became much more abundant on the streptomycin agar, but the colonies transferred to plain agar grew poorly, sometimes failing to grow on the second transfer. Round forms still predominated on the agar slant transfers, but rod forms appeared more frequently. The abundantly growing organisms on agar containing streptomycin tended to be less bizarre, and very few round forms were present.

Since attempts to obtain the variant form a second time from the parent culture under the stimulus of streptomycin had failed, it was thought that a similar variant might be selected from the reverted rod obtained by transfer of the round form on plain agar. As this series of transfers was made, each culture

was streaked on agar containing streptomycin and occasionally also on plain agar; colonies were selected from these plates, transferred to slants, and examined. At first it appeared that the changing of reverted rod forms back to round forms was being demonstrated since all colonies selected from the streptomycin plates gave rise to round-cell cultures, whereas increasing numbers of the colonies selected from plain agar were rods. But as the series continued with fewer and fewer round forms being present in the transferred culture, it was observed that fewer and fewer colonies appeared on the streptomycin plates at all, although they all continued to be the atypical cells. It was found that the cultures consisting of entirely reverted rods did not grow on streptomycin concentrations above 100  $\mu$ g per ml, whereas the round-cell cultures grew at 10,000  $\mu$ g per ml or, if selected from a lower concentration, grew at 10,000  $\mu$ g per ml on the second transfer to streptomycin. It could not be clearly demonstrated that reverted rod forms were being changed to round forms, but it appeared that the round forms remained resistant to high concentrations of streptomycin and that when they became rod forms they lost that resistance.

#### SUMMARY

A round-cell variant of *Escherichia coli* obtained from an agar plate containing streptomycin has been described. This was apparently a chance selection as no further similar strains were isolated. The variant and the parent strain gave identical biochemical reactions. Antigenically the variant and parent strain were not alike. The variant was maintained by alternate transfers on agar containing streptomycin and on plain agar. Serial transfers on plain agar produced a form which was identical to the parent strain culturally and morphologically. No evidence was found that the round-cell form was a part of a life cycle or a result of a sexual process.

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# THE ISOLATION OF TYPE B BOTULINUM TOXIN

CARL LAMANNA AND HAROLD N. GLASSMAN

*Camp Detrick, Frederick, Maryland*

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High yields of toxin have not been obtained with the nonproteolytic, and most of the proteolytic, strains of *Clostridium botulinum*, type B, in our culture collection. An exceptionally toxic type B culture designated as strain "okra" was received from the National Institute of Health. It is a proteolytic strain capable of producing, under certain conditions, one million minimum lethal doses of toxin for the mouse per ml of medium. From cultures of the "okra" strain a highly toxic and immunologically distinct protein has been isolated. Though this protein has not been crystallized it appears to be essentially a highly purified, single substance. The method of isolation for crystalline type A botulinum toxin of Lamanna, McElroy, and Eklund (1946) has not been applicable. This is probably a reflection of the physical and chemical differences that exist between the two serological types of toxin. Our methods and observations with the type B toxin are recorded in the following sections.

*Maintenance of the stock culture.* The stock culture is kept in chopped-beef infusion medium consisting of meat fragments submerged in a double-strength beef infusion including 0.5 per cent sodium chloride and 1 per cent Difco proteose peptone. Once a month the culture is transferred using a 1-ml inoculum per test tube. Incubation is at 34 C for 48 hours, and storage is at room temperature.

*Medium and growth for toxin production.* The organism is grown in 16-liter lots in 5-gallon pyrex glass carboys. The medium is composed of 1 per cent technical grade casein, 1 per cent alkaline-treated cornsteep liquor (47 to 52 per cent solids), and 0.5 per cent technical grade glucose. The cornsteep liquor is a filtrate of raw cornsteep treated by the addition of 1 part of water to 11 parts of cornsteep, alkalizing with 40 per cent sodium hydroxide to pH 8.5, and heating at 65 C for a half hour. The casein is brought into solution by agitation at pH 9.5 or higher. The cornsteep liquor is added, and the pH adjusted to 7.2. The mixture is autoclaved at 120 C for 1 hour. Upon cooling, the proper amount of glucose solution, which has been autoclaved separately, is added.

A flask with 500 ml of the same medium is preheated to remove dissolved air, cooled, and inoculated with the contents of a test tube of a 1-month-old stock culture. After overnight incubation at 34 C, 10 ml are transferred to 500 ml of medium in flasks corresponding in number to the carboys to be used for toxin production. These flasks in turn are incubated at 34 C overnight and then are used as inoculum, one per carboy. The carboys are incubated for 2 weeks at 34 C.

As a consequence of vigorous fermentation during the first 24 hours of growth the pH drops rapidly to values of 5.3 to 5.5. The casein comes out of solution both as a sediment and as a thick, firm pellicle floating at the surface as a result

of the entrapment of gas bubbles. At the beginning of the third day a decrease in the evolution of gas and a rise in pH are noticeable. The pH continues to rise slowly to 6.3 to 6.5 and may reach a value as high as 6.7 in two weeks' time. The decrease in acidity is accompanied by digestion of the casein. The bulk of the casein becomes soft in consistency, friable, and smaller in quantity. By the time the pH has risen to 6.0, toxin production in quantity becomes evident. A maximum titer (1 million MLD per ml or greater) is generally obtained within 10 days of incubation. No exact time for maximum accumulation of toxin can be stated as it has varied for each batch studied. As long as the pH does not rise above 6.5, there seems to be little loss of toxicity. Some batches have been incubated for 22 days without reduction in titer. The digestion of casein proceeds beyond the period of maximum toxin production and is presumably responsible for the continual decrease in acidity. It is of some advantage in the purification procedure to get rid of as much of the casein as possible by means of the natural proteolysis.

*Determination of toxicity.* The toxicity of all preparations is determined by intraperitoneal injection of 0.5-ml quantities of solution into 20-gram white mice ( $\pm 2$  g). The mice are observed for a period of 4 days. The term MLD is used in this report to signify the least amount of material killing all mice injected (usually 4 mice per dilution).  $LD_{50}$  is calculated by the method of Reed and Muench (1938) and is based on the use of 6 to 10 mice per dilution.

In making up dilutions of toxic solutions for titration purposes, 0.2 per cent gelatin buffered at pH 6.5 by the use of 1 per cent phosphate salts has been employed as diluent. It is a peculiarity of the toxin that it is relatively insoluble at pH values above 4.5. Therefore, to keep the toxin in solution during dilution, if a solution contains more than  $5 \times 10^6$  MLD per ml, it is more satisfactory to dilute with acidified water in the lower dilutions and with gelatin diluent in the higher dilutions.

Flocculation tests with commercially available horse antitoxins were studied briefly and discarded as an impractical means of following toxin concentration. The chief reason for this lies in the relative insolubility of the toxin at pH values greater than 4.5. Inasmuch as flocculation tests are generally performed near the neutrality point, the appearance of a flocculus with the toxin may be an index of loss of solubility rather than of a specific serological reaction. It is highly probable that the nature and quantity of other proteins present affect the solubility of this toxin. Depending on the stage of purity and the character of the serum in use, flocculation could, therefore, at times be the result of loss in solubility and at other times the result of the formation of specific toxin-antitoxin aggregates. Combined with our meager knowledge of the toxin-antitoxin reaction for the particular system, this complex state of affairs made the flocculation test a less practical means of following toxin concentration than titration in the mouse.

#### ISOLATION PROCEDURE

The method developed for the isolation of the toxin from the culture medium is fundamentally a series of acid precipitations. Salting out was tried at various

stages, but it was found to offer no advantages over acid precipitation of the toxin from solution. Slight additions of salt, such as 1 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  or NaCl to 0.1 M concentration, result in large decreases in solubility. This is different from the experience with type A toxin of Lamanna, Eklund, and McElroy (1946). The following is a description of the procedure. Unless noted otherwise, work was conducted at room temperature.

*Step 1: Acid precipitation of toxin from culture medium.* The 14-day-old cultures are acidified with a strong acid (2 N HCl) to pH 4.0. As a result the bacterial cells, undigested casein, toxin, and other acid-precipitable material slowly settle out. The carboys are permitted to stand overnight for the purpose of collecting as much of the insoluble matter as possible. The supernatant is siphoned off, and the acid precipitates are pooled and freed of liquid by centrifugation (1,800 rpm in 250-ml cups). The recovery of toxin in the acid mud may be 90 per cent or more effective. With some batches of culture pH 4.0 has not given quantitative recovery. It must be emphasized that the solubility of the toxin is probably greatly influenced by other proteins and products of bacterial growth. Inasmuch as the bacterial culture is a dynamic system, and all the variables affecting growth and proteolysis are not rigidly controlled, differences in composition between batches may exist at the time of acid precipitation of the cultures. Be this as it may, the empirically established fact is that an acid pH value exists at which the solubility of the toxin in the culture medium is at a minimum. This value is pH 4.0, or a neighboring one. Snipe and Sommer (1928) and Sommer (1937) made the original observation that botulinus toxins are precipitated from medium by acid.

*Step 2: Extraction of the toxin from the acid mud.* The precipitate from step 1 is resuspended in distilled water to one-fortieth the volume of the mother culture. This reduced volume will be referred to as the original volume. A strong acid (2 N HCl) is added to bring the pH to 2.0. As much as possible of the undissolved material is centrifuged off. The remainder is removed by filtration through a filter paper of fine porosity, which was previously wet with water acidified to pH 2. The clear filtrate contains about 80 per cent of the toxin.

At this stage, before filtration but after centrifugation, if the suspension is rotated an anisotropic appearance is noted. Under the microscope the suspended material is observed to be a mixture of amorphous and extremely small needle-shaped material. It gives typical chemical tests for a protein. The specific gravity of this material must be nearly that of the suspending solvent, for it can be removed only with great difficulty by repeated high-speed centrifugation. A Sorval angle centrifuge rotating at 13,000 rpm or the multispeed attachment of the International Equipment Company's refrigerated centrifuge (model PR-1) has been used. The anisotropic material is insoluble at all pH values studied (2.0 to 7.0). It remains finely divided at pH 2.0 to 4.5, aggregates above 4.5, and stays so up to pH 7.0, the highest pH value studied. This phenomenon is curious because the purified toxin itself in fairly concentrated solution remains soluble up to pH 4.5 and comes out of solution in clumps at higher pH values. The anisotropic material is not considered to be toxin since its potency per milligram of nitrogen is considerably lower than that for the

purified toxin, but it is definitely toxic. Repeated washing with acidified water does not entirely remove its toxicity. Thus, if the material itself is not toxic it strongly adsorbs toxin. The color of this material is nearly white in contrast to the purified toxin which has a grayish-yellow cast. The material must be associated with the growth of the organism, inasmuch as it is not obtainable upon similar treatment of unautoclaved and autoclaved medium in which the organism has not been grown.

The choice of distilled water acidified to pH 2 for redissolving the toxin from the acid precipitate of culture rests on a series of experiments with the toxic acid-precipitated mud which showed: (a) The toxin was rapidly detoxified at

TABLE 1

*Influence of pH, glycine, and salt on the resolution of toxin from an acid precipitate of medium*

pH	SALT ADDED (FINAL CONC.)	DILUTIONS (IN MILLIONS) OF SOLUTION KILLING 20-GRAM MICE			
		10	20	30	40
2.0	0	4/4 (26)*	2/4 (26) 2/4 (41)	4/4 (41)	2/4 (48) 1/4 (68)
	glycine 0.1 M	3/4 (26) 1/4 (41)	2/4 (26) 2/4 (41)	1/4 (41) 2/4 (48)	1/4 (48)
	NaCl 0.1 M	4/4 (26)	2/4 (26) 2/4 (41)	3/4 (41) 1/4 (48)	1/4 (48)
3.0	0	4/4 (26)	2/4 (26) 2/4 (41)	4/4 (41)	0/4
	glycine 0.1 M	3/4 (26) 1/4 (41)	3/4 (49) 1/4 (69)	0/4	0/4
	NaCl 0.1 M	0/4	0/4	0/4	0/4

\* Ratio =  $\frac{\text{no. of mice dying}}{\text{no. of mice injected}}$

Figure in parentheses is time in hours of observed death of mice.

pH values above 6.5. Thus, a suspension of  $20 \times 10^6$  MLD per ml lost none of its potency in 3 days at room temperature at pH values of 2, 3, 4, and 5. At pH 6.5 less than 10 per cent was lost; at pH 7.5 there was a loss of about 80 per cent; and at pH 8.5 there was more than a 90 per cent loss. (b) Resolution was poor in the pH range 4.5 to 6.5 and was quantitative only below pH 4. A significant difference existed even at pH 3 and 2 (table 1). (c) The addition of sodium chloride or glycine to the extracting solvent decreased the amount of toxin going back into solution, the effect being more notable at the higher pH values (table 1).

*Step 3: Precipitation of toxin from the extract of acid mud.* The clear filtrate



of step 2 is brought to pH 4.0 by slow addition of 2 N NaOH and cooled to refrigerator temperature. A flocculent precipitate, which is collected by centrifugation, forms.

Recovery of toxin in this step has varied considerably. As the toxin is purified, it seems to become progressively more soluble at pH values on the acid side of the isoelectric range. This would definitely point to impurities as influencing the solubility of the toxin. If, because of variations between cultures, the nature and quantities of impurities vary, then the differences in recovery of toxin from separate batches at this stage are explicable. More recently it has been found that precipitation at pH 5.0 will result in higher and more consistent yields.

*Step 4: Washing of the precipitate of step 3.* The precipitate of step 3 is washed by resuspension for 5 to 10 minutes in one-fourth original volume of a solution of 1.5 M NaCl at pH 2.0. The washed toxin is recovered by centrifugation. The solubility of the toxin in this solvent is low, being less than 100,000 MLD per ml.

*Step 5: Reprecipitation of the toxin.* The centrifuged material of step 4 is redissolved in one-fourth original volume of acidified distilled water at pH 2.0. Difficulty with resolution of the toxin will be experienced if much sodium chloride is carried over from the preceding step. The toxin is then reprecipitated by bringing the solution to pH 5.0 to 5.5 and collected by centrifugation.

The precipitate of step 5 represents purified toxin. It should be noted that except for the original toxic culture medium the solutions of the toxin are worked with at pH values on the acid side of the isoelectric zone. The method permits recovery of 50 per cent or more of the toxin.

#### PROPERTIES OF PURIFIED TOXIN

The purified toxin is an odorless, slightly grayish-yellow-colored solid. Solutions appear yellow brown in color. During electrophoresis the color travels with the toxin boundary and shows no tendency to separate from it. No characteristic absorption spectrum is shown in the range from 800 to 340  $m\mu$ . It reacts positively in qualitative tests for protein such as the biuret, ninhydrin, Millon's, xanthoproteic, and Hopkins-Cole. The Molisch test for carbohydrate is negative. The phloroglucinol test for nucleic acid is negative, and the orcinol test (Bial's reagent) slightly positive or negative. The absorption spectrum with ultraviolet light shows no evidence of the presence of nucleic acid. Absorption is maximum at 277  $m\mu$ . Extinction plotted against wave length gives a curve typical for a simple protein (figure 1). Chemical analyses for iron and metals precipitable by  $H_2S$  were negative. Thus no evidence for the presence of a prosthetic group has been obtained. The microkjeldahl nitrogen of toxin dried at 120 C in a vacuum oven is 15.5 per cent. Amide nitrogen has not been detected. Nitrogen of free amino groups was found to be 5.9 per cent of total nitrogen.

An estimate of molecular size was made using the Northrop and Anson (1929) sintered glass membrane diffusion apparatus and their method of calculation. The cell constant was determined by the use of NaCl. The diffusion constant

was calculated from analyses for nitrogen diffusing from the cell at 20 C in 12-hour intervals. The toxin was dissolved in acidified water at pH 2. The diffusion constant was  $0.0624 \text{ cm}^2$  per day or  $7.22 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ . Assuming a spherical shape this indicates a radius of  $2.9 \times 10^{-7} \text{ cm}$  and a molecular weight of about 60,000. This is in contrast to 900,000, the figure obtained by Putnam *et al.* (1946), and 1,200,000, obtained by Kegeles (1946) by different methods, for the molecular weight of crystalline type A toxin.

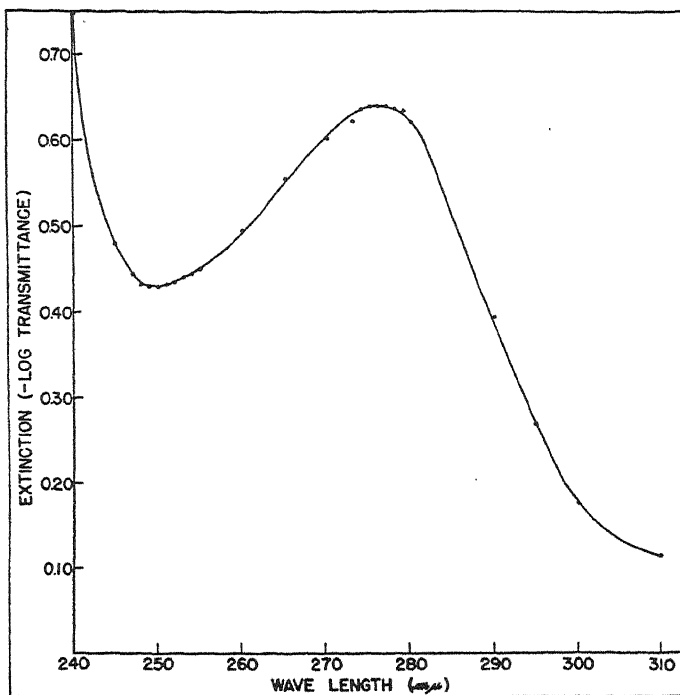


FIG. 1. ULTRAVIOLET LIGHT ABSORPTION SPECTRUM OF PURIFIED TYPE B TOXIN

It is difficult to place the toxin in the American system of classification of the proteins. On heating, it is denatured and will coagulate above pH 4.5, but it does not have easily classified solubility properties. Electrophoretic mobility shows that pH 4.5 and lower values represent the acid side of the isoelectric range. The toxin is quite soluble in water on the acid side of its isoelectric range and only slightly soluble on the alkaline side. At pH 5.0 and 5.5 at 7 C, 0.043 mg of toxin nitrogen per ml was found in solution. At 20 C at pH 6.0, only 0.056 mg of nitrogen per ml was present. To date no electrophoretic studies between pH 4.5 and 7.0 have been possible because of inability to get sufficient material into solution.

Unlike the globulinlike type A toxin, purified B toxin in water suspensions does not significantly increase in solubility upon the addition of salts. On the other

hand, addition of NaCl to the mother culture can increase the solubility of impure toxin. Table 2 shows that increasing additions of NaCl to the medium after toxin production has occurred results in an initial rise in solubility, followed by a decrease. At the same time, the pH of minimum solubility shifts to more acidic values. Thus without addition of salt the minimum solubility is at pH 4.0, but with 2 M NaCl pH 3.0 is the point of minimum solubility. The changes in solubility that are being measured in the case of table 2 represent toxicities of a few hundred thousand LD<sub>50</sub> per ml, that is, extremely small concentration

TABLE 2

*Influence of NaCl on solubility of toxin in mother culture at three pH values*

SALT ADDED (FINAL CONC.)	pH	TOXICITY OF DILUTIONS (IN THOUSANDS) OF SUPERNATANTS AFTER CENTRIFUGING OUT INSOLUBLE MATTER IN CULTURE				
		50	100	200	400	800
0	4.5	1/2*	3/4	2/4	0/4	0/4
	4.0	0/2	0/4	0/4	0/4	0/4
	3.0	1/2	4/4	4/4	2/4	0/4
0.1 M	4.5	2/2	4/4	3/4	4/4	0/4
	4.0	2/2	3/4	1/4	0/4	0/4
	3.0	2/2	3/4	4/4	4/4	0/4
0.5 M	4.5	1/2	4/4	4/4	3/4	0/4
	4.0	1/2	4/4	4/4	1/4	0/4
	3.0	2/2	4/4	4/4	2/4	4/4
1.0 M	4.5	2/2	4/4	3/4	4/4	2/4
	4.0	2 2	4/4	2/4	0/4	0/4
	3.0	2/2	4/4	4/4	4/4	0/4
2.0 M	4.5	2/2	4/4	4/4	3/4	0/4
	4.0	2/2	2/4	0/4	0/4	0/4
	3.0	0/2	0/4	0/4	0/4	0/4

\* Ratio =  $\frac{\text{no. of mice dying}}{\text{no. of mice injected}}$ .

differences. A milliliter of solution of 100,000 LD<sub>50</sub> contains  $5 \times 10^{-4}$  mg of toxin nitrogen or a concentration of toxin of approximately 0.0003 per cent.

Electrophoretic studies in glycine buffer at pH 1.8 reveal a single boundary on the ascending side. In addition to the major boundary a small boundary, which rapidly moves off the field of view, occurs on the descending side and does not reappear on reversal of the current (figure 2). Electrophoresis at pH 3.8 in glycine buffer showed a single moving boundary; the fast-moving boundary noted on the descending side at pH 1.8 was not seen. Whether the latter is an anomaly or has some special significance remains to be determined.

*Identity and potency of toxin.* Toxin in culture medium and purified toxin were typed by using commercially available horse antitoxins in mouse protection

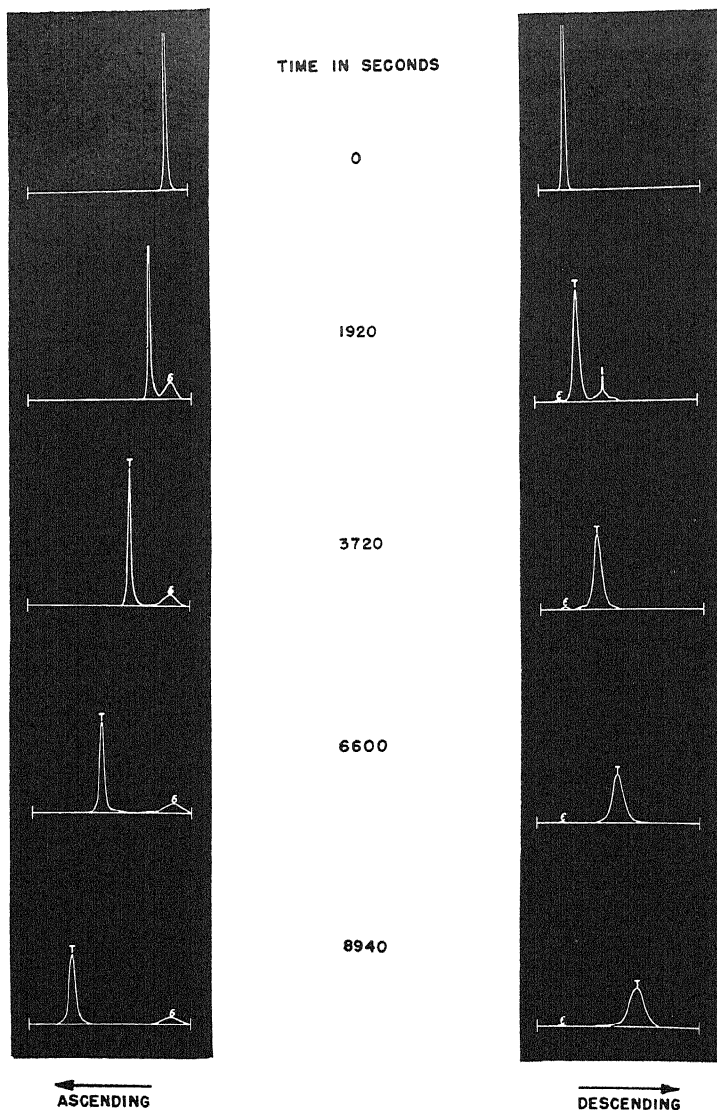


FIG. 2. ELECTROPHORETIC PATTERN OF PURIFIED TYPE B BOTULINUM TOXIN (CONC. 0.64%) DISSOLVED IN 0.1 M GLYCINE-HCl BUFFER AT PH 1.8 AT 3.5 C

The moving boundary marked T is the toxin. At 1,920 seconds the measurement of mobility was  $10.1 \times 10^{-5}$  cm<sup>2</sup>/sec/volt on the descending side and  $11.6 \times 10^{-5}$  cm<sup>2</sup>/sec/volt on the ascending side. Note a second moving boundary on the descending side labeled 1 in the 1,920 seconds' photo. This boundary rapidly moved off the field of view so that it is no longer visible in the 3,720 seconds' photo. Its mobility was  $25.3 \times 10^{-5}$  cm<sup>2</sup>/sec/volt. For this run the field strength was 4.31 volts per cm and the current 25 milliamperes. The stationary delta and epsilon boundaries are marked accordingly. The illustration is a composite of drawings made from tracings of the original photographs.

tests. Monovalent types A and C antisera did not protect, but type B monovalent antisera did. Rabbit antisera available as a result of immunization with

crystalline type A toxin, or toxoid prepared from crystalline toxin, showed no protective activity. Immunologically, there can be no doubt as to the identity of the toxin produced by the strain "okra."

On titration of one batch of the toxin in the mouse and the guinea pig, the following values were obtained:

$$\begin{aligned}6.2 \times 10^{-9} \text{ mg nitrogen} &= \text{LD}_{50} \text{ per 20-g mouse;} \\31.2 \times 10^{-9} \text{ mg nitrogen} &= \text{LD}_{50} \text{ per 300-g guinea pig;} \\310 \times 10^{-9} \text{ mg nitrogen} &= \text{LD}_{50} \text{ per kilo mouse;} \\103 \times 10^{-9} \text{ mg nitrogen} &= \text{LD}_{50} \text{ per kilo guinea pig.}\end{aligned}$$

These figures show that on a body weight basis the guinea pig is only three times as sensitive to the toxin as the mouse. This conclusion is in sharp contrast to the recent report of Stevenson, Helson, and Reed (1947) that the guinea pig is 6,000 to 8,000 times as sensitive as the mouse to the type B toxin. These workers have used strains other than "okra" and relatively impure toxin. The identity of similar serological types of toxin from different bacterial strains has been assumed, but final proof will rest with studies on pure materials isolated from different strains.

Separately prepared batches of purified toxin have given values of toxicity from 5 to  $9 \times 10^{-9}$  mg of nitrogen per mouse  $\text{LD}_{50}$ . This is of the same order of magnitude as  $4.5 \times 10^{-9}$  mg nitrogen obtained with crystalline type A toxin. On a weight basis the two toxins appear equipotent; but if future research confirms the finding that the B toxin is 10 or more times smaller in molecular size, then on a molar basis the B toxin is considerably less toxic.

The stability of the purified B toxin has presented itself as a serious problem. It appears more labile than the impure material. As a result we have not succeeded in storing solutions in the refrigerator for periods greater than two weeks without serious losses in potency. Interestingly enough, one batch of reduced potency studied appeared to have unaltered electrophoretic properties at pH 2.

#### ACKNOWLEDGMENT

The authors are indebted to Dr. J. W. Hornibrook of the National Institute of Health, who made available to us the "okra" and other strains of the botulinus organism. Grateful acknowledgment is made to Mr. John H. Convey for the electrophoretic examinations and to Mr. C. A. Grabill and Mr. H. H. Moorefield for technical assistance.

#### SUMMARY

A method for the purification of the type B toxin from the proteolytic "okra" strain of *Clostridium botulinum* is described. Essentially the purification depends upon working with the toxin on the acid side of its isoelectric zone and upon a series of acid precipitations. The purified toxin appears to be a slightly colored, simple protein, soluble in water on the acid side of the isoelectric range and relatively insoluble on the alkaline side and within the isoelectric range. Slight additions of salt do not favor increased solubility of the purified toxin.

Serologically, chemically, and physically the purified B toxin differs from type A crystalline toxin. Its toxicity per milligram of nitrogen is only slightly less than that of the type A, but on a molar basis, it would appear to be 10 times less potent. By the intraperitoneal route the guinea pig is about three times more susceptible to the toxin than is the white mouse.

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# STUDIES ON POLYMYXIN: AN AGAR DIFFUSION METHOD OF ASSAY

P. G. STANSLY AND M. E. SCHLOSSER

*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

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*Bacillus polymyxa* was originally detected as a producer of an antibiotic (Stansly, Shepherd, and White, 1947) by the fact that surrounding the colony of the organism was a clear zone of agar, whereas the remainder of the agar plate was covered with a layer of growth of the test organism. Obviously the antibiotic diffused through the agar and prevented growth of the test organism up to a point where its concentration fell to a value below that required for this inhibition. It was, therefore, anticipated that it might be possible to develop an agar diffusion method to assay for potency similar to the well-known methods for penicillin (Abraham *et al.*, 1941).

It was soon apparent, however, that the methods used for the assay of penicillin were not strictly applicable to the assay of polymyxin. For example, in a preliminary experiment using filter paper disks saturated with solutions of polymyxin, insignificant zones of inhibition were obtained using *Escherichia coli* as the test organism, even with high concentrations of the antibiotic. Incubation of the plates was at 37 C. Experiments were then undertaken, many of them qualitative in nature, to determine the conditions necessary for obtaining large, sharply defined inhibition zones. When these conditions were determined, it was anticipated that it might then be possible to relate zone size to antibiotic concentration.

The following variables were studied: (1) the type of medium in base and seed layers, (2) the concentration and amount of agar in the base and seed layers, (3) the pH of the medium in the seed layer, (4) the incubation time and temperature, (5) the effect of surface-active agents, (6) the pH of antibiotic solutions, (7) the type of test organism, (8) the inoculum size in the seed layer, and (9) the use of filter paper disks as opposed to ceramic cylinders. In all of these studies with the exception of that involving test organisms, *E. coli* (MacLeod) was the assay organism used.

Brief statements of the results obtained will be followed by an account of the final method which evolved and the type of data which was obtained. A statistical analysis of the assay and a method for estimating the error of any assay will be given.

*The type of medium in base and seed layers.* A variety of media and combinations of nutrients at different concentrations were tried. Few were equal to and none superior to "TSP"<sup>1</sup> (at a concentration recommended by the manufacturer)

<sup>1</sup> Trypticase-soy-phosphate medium (Baltimore Biological Laboratory).

from the standpoint of zone size and definition. Since, in addition, "TSP" is a ready-made dehydrated material, it was selected as the assay medium of choice.

*Concentration of agar and amount of agar in base and seed layers.* To favor diffusion in the seed layer it was thought desirable to reduce the agar concentration to a minimum. At first 1.5 per cent agar was used and later 1.2 per cent. Four ml of agar was considered to be the smallest amount that could be conveniently spread over the base layer. The base layer was more or less arbitrarily set at 20 ml of 2 per cent agar. Some variation of this was tried but resulted in no particular advantage.

*pH of medium in seed layer.* A comparison of zones obtained with the seed layer adjusted to an initial pH of 5, 7, and 9 was made. Growth but no zones were obtained at pH 5. The zones at pH 9 were smaller than those at pH 7. The pH of the seed layer medium was therefore set at 7.

*Incubation time and temperature.* The importance of the proper incubation conditions for the success of the assay cannot be overemphasized. It is worth repeating that incubation at 37 C, no matter what other conditions were imposed, led only to insignificant zones of inhibition even with high antibiotic concentrations. It was surmised that these insignificant zones at 37 C were due to one of two factors or, perhaps more correctly, to an interaction of the two. These factors were, first, and possibly most important, the extremely rapid growth rate of *E. coli* (Mason, 1935) and, second, the relatively slow diffusion of polymyxin.

To combat the first, recourse was had to lower incubation temperatures. This had the desired effect of increasing markedly the zone of inhibition. However, no single incubation temperature between 15 C and 30 C was entirely satisfactory because the depressing effect on growth resulted in poorly defined and uneven zones. On the other hand, an initial period of low temperature incubation followed by a period of higher temperature incubation was found to be a satisfactory compromise. It appeared that the low temperature incubation slowed down bacterial growth to such an extent that the relative rates of growth of *E. coli* and of diffusion of the antibiotic were in favor of the latter. After the antibiotic had been given an opportunity to diffuse before appreciable growth started, it was possible to continue incubation of the plates at a higher temperature without adversely affecting the zone of inhibition. The 37 C incubation may be looked upon as merely a device for smoothing and accentuating the contrast at the edge of the zone by providing favorable conditions for the rapid multiplication of the bacteria surrounding it. As finally evolved, the low temperature incubation was at 25 C for 18 hours. This was followed by 6 hours at 37 C, thus permitting an assay to be completed in 24 hours.

*Effect of surface-active agents.* The second factor influencing zone size was considered to be the diffusion of the antibiotic. It was thought that substances which reduce interfacial tension might increase diffusion, thus increasing the zone size and hence the sensitivity of the assay. The following experiments illustrate the results obtained. All measurements were an average of three replicate zones.

*Experiment 1.* "Aerosol OT"<sup>2</sup> and "tween 60"<sup>3</sup> were incorporated in the seed

<sup>2</sup> American Cyanamid Company.

<sup>3</sup> Atlas Powder Company.



layer at various concentrations. All other conditions were the same and need not be described here. The results are shown in table 1.

Table 1 shows that "tween 60" had the effect of increasing the size of the zone. On the other hand, "aerosol OT" failed to do this. Of incidental interest is the apparent antagonistic effect of "aerosol OT" on the antibiotic. In the concentrations used, neither "aerosol OT" nor "tween 60" had any observable effect on the growth of *E. coli*.

TABLE 1

*Effect of "aerosol OT" and "tween 60" on the zone of inhibition in the assay of polymyxin*

CONC. REAGENT	ZONE DIAMETER IN MM	
	Aerosol OT	Tween 60
<i>per cent</i>		
0.5	0.0	26.75
0.05	0.0	25.5
0.005	18.0	23.0
0.0005	18.0	22.25
0.00005	22.0	
0.0	22.0	

TABLE 2

*Effect of "tween 60" and "tween 80" on zone of inhibition in the assay of polymyxin*

CONC. REAGENT	ZONE DIAMETER IN MM	
	Tween 60	Tween 80
<i>per cent</i>		
4.0	30.0	30.0
2.0	30.0	29.0
1.0	30.0	29.25
0.5	29.25	29.0
0.25	29.25	29.0
0.125	30.0	29.0
0.05	28.5	29.0
0.005	27.5	28.0
0.0005	27.25	27.25
0.00005	27.0	27.5
0.0	26.5	

*Experiment 2* is a comparison of "tween 60" and "tween 80"<sup>4</sup> under identical conditions. The results are summarized in table 2, which confirms the general effect noted in experiment 1. "Tween 60" and "tween 80" were approximately equivalent in activity.

*Experiment 3* is a titration of polymyxin comparing the zones obtained with "tween 80" (1 per cent) and those obtained in the absence of any surface-active agent. Table 3 indicates the results. These confirm the results of experiments 1 and 2 and, in addition, demonstrate the increased sensitivity obtained with the

<sup>4</sup> Atlas Powder Company.

surface-active agent. That is, under these conditions 8 units per ml could be detected in the presence of "tween 80," whereas only 16 could be determined in its absence.

TABLE 3  
*Titration of polymyxin with and without "tween 80"*

CONC. POLYMYXIN  units/ml*	ZONE DIAMETER IN MM	
	Control	Tween 80
128	26.7	28.8
64	24.3	25.7
32	19.7	23.25
16	16.7	20.0
8	0	16.0
4	0	0

\* For a definition of the unit see "Procedure."

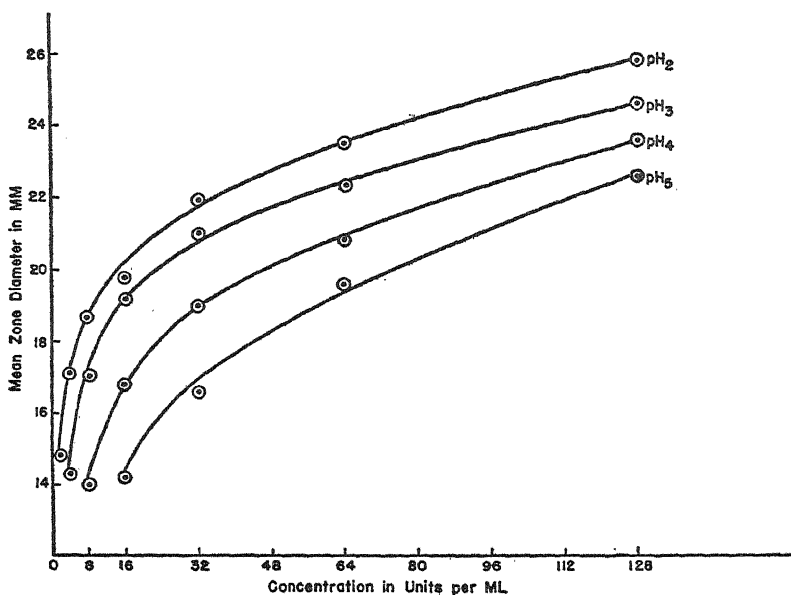


FIG. 1. EFFECT OF pH OF POLYMYXIN SOLUTIONS ON ZONE DIAMETERS

*Effect of pH of antibiotic solutions.* Up to this point, solutions of polymyxin for the purpose of potency determinations were made up in 0.02 M phosphate buffer, pH 7.0. It was now desired to determine the effect, if any, of varying the pH of such solutions upon the quality and diameter of the inhibition zones.

*Experiment 1.* Solutions of the antibiotic were prepared in 0.85 per cent saline adjusted to pH 3, 5, 7, and 11 and diluted from 512 units per ml to 2 units per ml in saline of the respective pH. The zone diameters were approximately equivalent at corresponding concentrations for the solutions of pH 5, 7, and 11, but

were definitely larger at pH 3. Four units per ml gave a measurable zone in the latter instance, whereas 8 units per ml were required for the other preparations.

*Experiment 2.* Solutions of polymyxin were made up in and diluted with glycine-HCl buffers<sup>5</sup> at pH 2, 3, 4, and 5, and assayed. The results are best illustrated graphically and are shown in figure 1. In this experiment it was possible to determine 2 units per ml of polymyxin in solution at pH 2, whereas only 16 units per ml could be determined at pH 5. The buffers alone had no apparent effect on the growth of the test organism.

Solutions of polymyxin are quite stable except in alkaline regions (Stansly *et al.*, 1947). Therefore it seems unlikely that the effect of the pH of the antibiotic solution on zone size is a reflection of pH stability of the antibiotic. These effects are likewise unrelated to any interaction with the filter paper of the disks since the same effects were demonstrated with ceramic cylinders. The underlying reason for the apparent increased diffusion or activity of polymyxin in the agar medium with decreasing pH is at present obscure.

In addition to increasing the sensitivity of the assay, another advantage was apparent with antibiotic solutions at the lower pH's in the glycine-hydrochloric-acid buffers. The zones appeared to be more consistently round and regular than those obtained with the same material at pH 7 in phosphate buffer or saline.

*The test organism.* A desirable test organism for the assay is one which is very sensitive to the antibiotic, gives good growth in 24 hours, and is preferably non-pathogenic. *E. coli* (MacLeod) satisfied all these requirements. However, it seemed worth while to look for an organism with such a growth rate that the entire assay could be carried out at a single temperature. Several were investigated (e.g., *Salmonella pullorum*), but none proved satisfactory for this purpose.

*Effect of inoculum size.* The smaller the number of bacteria in the seed layer, the larger the resulting zone of inhibition, and hence the greater the sensitivity of the assay. If carried far enough, however, a point is reached at which increased sensitivity can be achieved only at the expense of definition and regularity of the zones. The optimum inoculum was obtained by diluting a 24-hour, 37 C broth culture of *E. coli* (MacLeod) to a final concentration of 1:2,000 in the agar seed layer.

*Filter paper disks vs. ceramic cylinders.* No differences were found between disks and cylinders other than that ascribable to the differences in their respective diameters. Thus, a zone obtained with a given concentration of polymyxin measured 25 mm with the disk and 21 mm with the cylinder, but the difference in the diameters of the disk and cylinder was exactly 4 mm. From the standpoint of simplicity and convenience, the disk seemed far superior to the cylinder method and was selected as the procedure of choice.

#### PROCEDURE

*The standard.* Lot 5 (crude, dry acetone precipitate) was set aside as the standard preparation of polymyxin for assay purposes and stored in a desiccator (under calcium chloride) in the refrigerator.

<sup>5</sup> Sorensen's buffer mixtures (Gortner's *Outlines of Biochemistry*, 2d ed., p. 123). This buffer at pH 2 was also used for the routine assay as described under "Procedure."

*The unit.* Repeated assay of the standard by the agar streak method gave an inhibition end point of 8 micrograms per ml with *E. coli* (MacLeod) as the test organism. Therefore, the unit of activity was considered as equivalent to the activity of 8 micrograms per ml of the standard preparation.

*Preparation of the standard solution.* The standard (204.8 mg) is dissolved in 100 ml of 0.05 M glycine-hydrochloric-acid buffer, pH 2, giving 256 units per ml. Tests have shown that such a solution is stable indefinitely in the refrigerator. Further dilutions of the standard are made in the same buffer.

*Preparation of samples for assay.* Solid samples are dissolved in 0.05 M glycine-hydrochloric-acid buffer, pH 2, and dilutions are made with the same buffer. Aqueous liquids (e.g., fermentation liquors) are first diluted with an equal quantity of 0.1 M glycine-hydrochloric-acid buffer, pH 2, and further dilutions are made with 0.05 M buffer. Nonaqueous solutions of polymyxin are preferably evaporated to dryness and then treated as solid samples. Insufficient experience with nonaqueous solvents or mixed solvents does not justify any statement of the validity of their use in the standard assay procedure.

*Preparation of plates.* Twenty ml of 2 per cent TSP agar (pH approximately 7.3) are poured into petri plates and allowed to solidify. Four ml of 1.2 per cent agar containing 1 per cent "tween 80" and a 1:2,000 dilution of a 24-hour, 37 C TSP broth culture of *E. coli* (MacLeod) are then spread over the base layer. The seed agar is dispensed with a 10-ml pipette from a single flask maintained at 48 C in a water bath. The plates are next dried in special trays, with lids raised, for 45 minutes in a dry 37 C incubator.

*Preparation of saturated disks.* Three filter paper disks (Schleicher and Schuell no. 740E,  $\frac{1}{2}$ " diameter) in a sterile petri dish are saturated with a single dilution (of either standard or unknown) by distributing 0.4 ml from a 1-ml pipette. Any excess is removed by touching the disk twice on a dry area of the plate. The saturated disks are then placed on the seeded agar by means of forceps. The distribution of the saturated replicate disks is dependent upon the manner of estimating potency, as will be brought out later.

*Incubation of completed plates.* The completed plates are replaced in the special trays mentioned above and incubated overnight (16 to 18 hours) at 25 C with lids raised. The following morning the trays and plates are transferred to a well-humidified 37 C incubator and incubated for 6 more hours. The plates are then taken out and the zone diameters measured in mm in any convenient way.

#### DETERMINATION OF POTENCY

*Method 1.* In this method three disks are saturated with a dilution of the standard solution and placed upon a seeded plate. This provides one point of the standard curve. Other points are obtained from similar plates containing additional dilutions of the standard. Only one dilution of the unknown is used. Three disks are saturated with it and also placed upon a seeded plate. Upon each plate, in addition, is placed a single disk saturated with one particular concentration of the standard, e.g., 128 units per ml, the purpose being to ensure

that all the plates are uniformly prepared. This would presumably be indicated by obtaining the same size zone on each plate from this particular disk.

The triplicate disks of each standard dilution are averaged, and a standard curve relating potency in units per ml (plotted logarithmically) to diameter of zone of inhibition is constructed. Such a standard curve is shown in figure 2. Fermentation liquors also give a linear relationship.

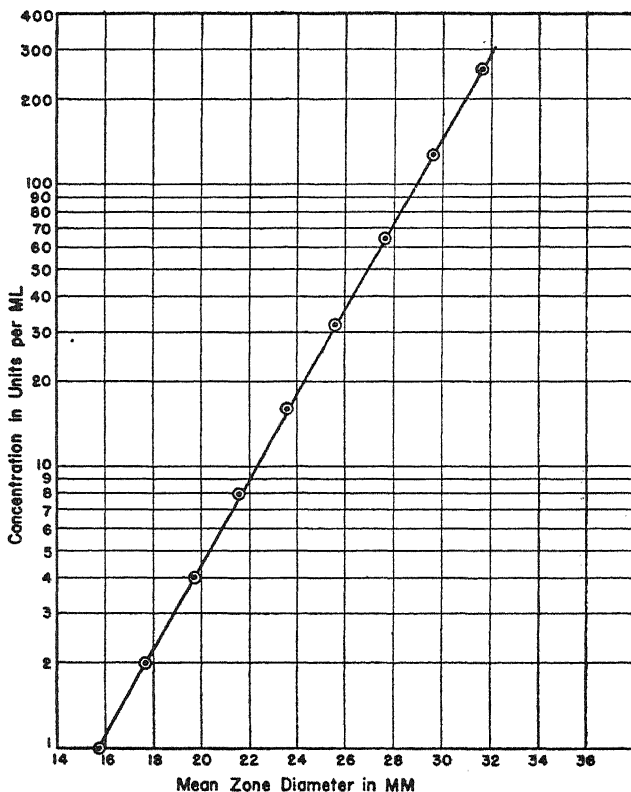


FIG. 2. STANDARD POLYMYXIN ASSAY CURVE

This method has been successfully used when a relatively small number of assays were to be made. Certain difficulties became apparent when a large number of assays were made because of the lapse of several hours between the time the first and last samples were completed. For example, suppose that the plates for the standard curve are prepared first and that the last sample is assayed after 1 hour. During this time growth could take place at an appreciable rate (depending on the room temperature) in all the plates, but diffusion of the antibiotic only in those plates already completed (including the standard plates). Therefore, the resulting standard curve could not be used for estimating the potency of the last sample without incurring a considerable error, since equal concentrations of antibiotic would give different zone sizes under these conditions.

The following experiment illustrates this type of behavior: Upon each of three seeded plates was placed a filter paper disk saturated with 64 units per ml of a standard solution. After standing at room temperature for an hour, each of the three plates received another freshly saturated disk of 64 units per ml of the standard solution. After the usual incubation the zone diameters were measured, with results given in table 4. This table shows that the average difference between the two treatments was 3.0 mm. If it were imagined that the standard applied after 1 hour was in reality an unknown sample, its potency would have been reported as about 33 per cent too low.

*Method 2.* To obviate the foregoing difficulty the method of the Food and Drug Administration for the assay of penicillin (Knudson and Randall, 1945) seemed admirably suitable. In this method each assay is independently performed in conjunction with its own standard. No standard curve in the usual sense is used. The results are calculated by the use of formulae or nomographs derived from consideration of the straight-line log dose vs. response relation-

TABLE 4  
*Effect of delay in applying saturated disk to seeded plate*

ZONE DIAMETER (MM) UPON APPLYING DISK	
Immediately	After 1 hour
26	23
27	24
27	24
Avg.....26.6	23.6

ship, such as exists with penicillin and polymyxin. In the procedure now in use, two disks of the standard, each saturated with a different concentration, and two disks of the unknown, also of different concentrations, are placed upon a single seeded plate. It is essential that the ratio of potencies of the two standard disks be the same as the two unknown disks. In our case the ratio is usually 4. Three replicate plates are used, rather than four as recommended for penicillin by the Food and Drug Administration.

A word should be said about the assay of fermentation liquors as opposed to that of concentrates. Concentrates invariably give zones of excellent definition. This is not always the case with fermentation liquors. Occasionally, and this seems to be particularly true of "stationary" fermentation liquors, diffuse or double zones, or both, are obtained which render measurement difficult and uncertain. The concentration process evidently removes the interfering substance. Fortunately aerated cultures are relatively free of this defect. When it does occasionally exist, it is usually of no serious consequence.

#### STATISTICAL ANALYSIS OF THE MICROBIOLOGICAL ASSAY OF POLYMYXIN

Since it was anticipated that the error in the assay of fermentation liquors would be greater than that in other samples (e.g., concentrates), the data were

divided into two categories. The first comprised clarified beers and the second all other types of preparations. Ten assays in each category were taken at random from over a period of a month and subjected to an analysis of variance, which took the form shown in table 5.

It is apparent that seven degrees of freedom per assay, derived from the interactions, were available for the estimate of error. The standard deviation, based on the total interactions of the ten assays (70 degrees of freedom) in each category, was found to be 0.434 mm for beers and 0.304 mm for all other samples. The  $\chi^2$  test revealed a significant difference between the two values.

TABLE 5  
*Form of analysis of variance for the assay of polymyxin*

SOURCE OF VARIATION		DEGREES OF FREEDOM
Type	Source	
Main effects	Standard vs. unknown (preparations)	1
	Low conc. vs. high conc. (concentrations)	1
	Replicate plates	2
First order interactions	Preparations $\times$ concentrations	1
	Preparations $\times$ plates	2
	Concentrations $\times$ plates	2
Second order interaction	Prep's. $\times$ conc's. $\times$ plates	2
Total.....		11

The standard error of a log ratio of potency,  $S_M = Skit \sqrt{\frac{B^2 + D^2}{B^2}}$  (Bliss and Marks, 1939), wherein

$S = 0.434$  or  $0.304$  mm

$k = 1$  (for a two-dose test)

$i = 0.602$  (log of the interval of doses, i.e., log 4)

$t = 1.994$  for 19 in 20 odds

$D^2 = \frac{V^2}{12}$ , where  $V = \Sigma(V_H + V_L) - (S_H + S_L)$  for the triplicate plates.

The symbols  $V_H$ ,  $V_L$ ,  $S_H$ , and  $S_L$  are the zone diameters of high and low unknown and high and low standard, respectively (Knudson and Randall, 1945).

$B^2 = \frac{W^2}{12}$ , where  $W = \Sigma(V_H + S_H) - (V_L - S_L)$  for the triplicate plates of an assay (Knudson and Randall, 1945).

Substituting these values,  $S_M = \frac{K \sqrt{V^2 + W^2}}{W^2}$ , wherein

$K^1 = 1.81$  (beers)

$K^2 = 1.27$  (all other samples).

$100 \left[ \text{antilog} \left( \frac{K \sqrt{V^2 + W^2}}{W^2} \right) - 1 \right]$  expresses the error of the assay as a per-

TABLE 6  
*Error of assay as a percentage of potency\**  
 (95 per cent probability for beers)

	$W \rightarrow 20$	21	22	23	24	25	26	27	28	29	30
±											
0	23	22	21	20	19	18	18	17	16	15	15
1	23	22	21	20	19	18	18	17	16	15	15
2	23	22	21	20	19	18	18	17	16	15	15
3	23	22	21	20	19	18	18	17	16	15	15
4	24	23	22	20	20	18	18	17	17	16	15
5	24	23	22	20	20	18	18	17	17	16	15
6	24	23	22	21	20	18	18	17	17	16	15
7	25	23	22	21	20	18	18	18	17	16	15
8	25	24	23	21	20	19	19	18	17	16	16
9	25	24	23	22	21	19	19	18	18	16	16
10	26	25	23	22	21	19	19	18	18	16	16
11	27	25	24	22	21	20	19	18	18	16	16
12	27	26	24	23	22	20	20	18	18	16	16
13	28	26	25	23	22	20	20	19	18	17	16
14	29	27	25	24	23	21	20	19	19	17	16
15	29	28	26	24	23	21	21	19	19	18	17
16	30	29	27	25	24	21	21	20	19	18	17
17	31	29	27	25	24	22	21	20	20	18	18
18	32	30	28	26	25	22	22	20	20	18	18
19	33	31	29	27	25	23	22	21	20	18	18
20	34	32	29	27	26	23	23	21	21	19	18
21	35	33	30	28	26	24	23	22	21	19	18
22	36	33	31	29	27	24	24	22	21	20	19
23	37	35	32	29	28	25	24	22	22	20	19
24	38	36	33	30	29	25	25	23	22	20	19
25	39	37	34	31	29	26	25	23	23	21	20
26	41	37	35	32	30	27	26	24	23	21	20
27	42	39	35	32	31	27	26	24	24	21	21
28	43	40	37	33	31	28	27	25	24	22	21
29	44	41	37	34	32	29	28	25	25	22	21
30	45	42	38	35	33	29	28	26	25	23	22
31	46	43	39	36	33	30	29	26	26	23	22
32	48	44	40	37	34	30	29	27	26	24	23
33	49	45	41	37	35	31	30	28	27	24	23
34	50	46	42	38	36	32	31	28	27	24	23
35	52	48	43	39	37	32	31	29	28	25	24
36	53	49	44	40	37	33	32	29	28	25	24
37	55	50	46	41	38	34	33	30	29	26	25
38	56	51	47	42	39	35	33	31	29	26	25
39	57	52	48	43	40	35	34	31	30	27	26
40	59	54	49	44	41	36	35	32	31	28	26

$$* 100 \left[ \text{Antilog} \left( \frac{1.81 \sqrt{V^2 + W^2}}{W^2} \right) - 1 \right].$$



TABLE 7

*Error of assay as a percentage of potency\**  
(95 per cent probability, all samples except beers)

V	$W \rightarrow 20$	21	22	23	24	25	26	27	28	29	30
$\pm$											
0	16	15	14	13	13	13	12	12	11	11	10
1	16	15	14	13	13	13	12	12	11	11	10
2	16	15	14	13	13	13	12	12	11	11	10
3	16	15	14	13	13	13	12	12	11	11	10
4	16	15	14	14	13	13	12	12	11	11	10
5	16	15	15	14	14	13	12	12	11	11	10
6	16	15	15	14	14	13	13	12	11	11	11
7	17	16	15	14	14	13	13	12	11	11	11
8	17	16	15	14	14	13	13	12	12	11	11
9	18	16	15	14	14	13	13	13	12	11	11
10	18	16	16	15	14	13	13	13	12	11	11
11	18	17	16	15	15	13	13	13	12	12	11
12	19	17	16	15	15	14	13	13	13	12	11
13	19	18	16	16	15	14	14	13	13	12	11
14	20	18	17	16	16	14	14	13	13	12	11
15	20	18	18	16	16	15	14	13	13	12	11
16	21	19	18	16	16	15	14	14	13	13	12
17	21	19	18	17	16	15	15	14	13	13	12
18	22	20	19	17	17	15	15	14	14	13	12
19	22	20	19	18	17	16	15	15	14	13	13
20	23	21	19	18	18	16	15	15	14	13	13
21	24	21	20	18	18	16	16	15	14	14	13
22	24	22	20	19	18	17	16	15	15	14	13
23	25	23	21	19	19	17	16	16	15	14	13
24	26	23	22	20	19	18	17	16	15	14	13
25	26	24	22	20	20	18	17	16	16	15	14
26	27	24	23	21	20	18	18	16	16	15	14
27	28	25	23	21	21	19	18	17	16	15	14
28	29	26	24	22	21	19	18	17	16	15	14
29	29	26	24	22	22	19	18	18	16	16	15
30	30	27	25	23	22	20	19	18	17	16	15
31	31	28	26	23	22	20	19	18	17	16	15
32	32	29	26	24	23	21	20	19	18	16	16
33	33	29	27	24	24	21	20	19	18	17	16
34	33	30	27	25	24	22	20	19	18	17	16
35	35	31	28	26	25	22	21	20	19	18	16
36	35	31	29	26	25	23	21	20	19	18	16
37	37	32	29	27	26	23	22	21	19	18	17
38	37	33	30	27	26	23	22	21	20	18	17
39	38	33	31	28	27	24	23	21	20	19	18
40	39	34	31	29	27	24	23	22	20	19	18

$$* 100 \left[ \text{Antilog} \left( \frac{(1.27 \sqrt{V^2 + W^2})}{W^2} \right) - 1 \right].$$

centage of potency. Tables were prepared, one for beers (table 6) and one for all other samples (table 7), in which the error (for odds of 19 in 20) as a percentage of potency was calculated for values of  $V$  from 0 to 40 and  $W$  from 20 to 30, covering perhaps 99 per cent of all combinations of  $V$  and  $W$  likely to occur. In practice  $W$  does not vary appreciably from 24, whereas  $V$  varies in accordance with the relative potency of unknown and standard. It may be pointed out that the factors  $V$  and  $W$  are easily obtained for each assay and are prerequisites for calculating the potency of an unknown, in addition to the error.

The determination of potency and the estimation of its error are based on assumed parallelism of the log concentration vs. the response curves of standard and unknown. In isolated instances this assumption may not be justified, and hence the estimation of potency and its error would be invalid. Therefore, a test for departure from parallelism, i.e., a significant interaction of preparations  $\times$  concentration, is made routinely for each assay. The variance of this interaction divided by the triple interaction variance gives the required factor for the test. If this ratio is 4.35 or greater, then there is only a 5 per cent chance that the standard and unknown slopes are really the same, and it is presumed that they are significantly different. The factor 4.35 is obtained from a table of "F" for 1 and 20 degrees of freedom.

For routine purposes the test for significance of slope difference may be simplified. Thus, it was calculated, on the basis of the considerations above, that if  $(S_H - S_L) - (V_H - V_L)$  is 4.0 or more for beers and 2.0 or more for all other samples, then a significant departure of parallelism between standard and unknown slopes is presumed to exist, and the particular assay is discarded. A small percentage of the assays fall in this class.

Experience has shown that the usual error for a 95 per cent probability is  $\pm 20$  per cent for beers and  $\pm 15$  per cent for all other samples. This error can be further reduced by repeating the assay. The average percentage of error thus obtained divided by the square root of the number of repetitions gives the percentage of error of the average potency.

From time to time it may be desirable to check the standard deviations upon which the error is based. Quality control methods (Knudson and Randall, 1945), when justified, would also be of value.

#### ACKNOWLEDGMENT

It is a pleasure to acknowledge our indebtedness to Dr. Frank Wilcoxon of the Stamford Laboratories for the statistical analysis and for the many hours generously given in discussion.

#### SUMMARY

The factors influencing *Escherichia coli* inhibition zones produced by the antibiotic polymyxin are considered. An agar diffusion method of assay is described and a statistical analysis presented. As customarily used, the error for a 95 per cent probability is in the neighborhood of  $\pm 15$  to 20 per cent. This can be further reduced, if desired, by appropriate replication.

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# THE RELATION BETWEEN OXYGEN CONSUMPTION AND THE UTILIZATION OF AMMONIA FOR GROWTH IN *SERRATIA MARCESCENS*

DOROTHY J. MCLEAN AND KENNETH C. FISHER

*University of Toronto, Toronto 5, Canada*

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The metabolic differences between assimilating and nonassimilating cells have been the subject of several recent investigations. In this connection a stimulation of glycolysis when ammonia is added to yeast has been shown by several authors (Runnstrom, Brandt, and Marcuse, 1941; Winzler, Burk, and du Vigneaud, 1944). Armstrong and Fisher (1947) have demonstrated a comparable increase in the rate of oxygen consumption by *Escherichia coli* when the assimilation of ammonia is taking place. It follows from these observations that it may be possible to determine the amount of glycolysis or carbon dioxide produced and the amount of oxygen consumed during the assimilation of known quantities of the nitrogen source.

It will be shown in the present work that in the bacterium *Serratia marcescens* the rate of oxygen consumption is also higher during the assimilation of ammonia than it is in the absence of such assimilation. Following the uptake of the last of the ammonia, as noted for *E. coli* by Armstrong and Fisher, the rate at which oxygen is consumed by the bacterium falls sharply to a lower rate, which is typical of resting cells. This lower rate is a definite percentage of the higher one regardless of how much growth has taken place. It is, therefore, permissible to calculate the resting rate which corresponds to each rate observed for the growing cells. Any oxygen consumed in excess of the amount expected for resting cells must then be associated with the assimilation of ammonia. This quantity of oxygen has been measured along with the quantity of ammonia actually assimilated.

## MATERIALS AND METHODS

*The preparation and maintenance of the bacteria.* The organism used in this investigation was the bacterium *Serratia marcescens* (*Bacillus prodigiosus*), American Type Culture Collection no. 990. It was maintained on a synthetic medium, modified from that used by Bunting (1940), having the following composition: glycerol 1.25 g, citric acid 4 g,  $K_2HPO_4$  9 g,  $MgSO_4 \cdot 7H_2O$  0.5 g, and  $NH_4Cl$  1 g, adjusted to pH 7 with NaOH, and made up to 1 liter with distilled water. Twenty-five g of agar were added and the medium was autoclaved at 15 pounds' pressure for 15 minutes.

The bacterial suspensions for the respiration experiments were prepared as follows: A slant was inoculated from 1 loopful of bacteria; it was incubated for

17 hours at 30 C (a preliminary experiment showed that this temperature gave better growth than 20 or 37 C); and the growth was then washed off into 0.07 M potassium phosphate buffer at pH 7. The suspension was made up to the desired concentration, about  $1 \times 10^9$  bacteria per milliliter, by the reflectometer (Libby, 1941).

*The measurement of oxygen consumption.* The rate of oxygen consumption was measured in a Warburg respirometer (Umbreit, Burris, and Stauffer, 1945) at 30 C, with air being used as the gas phase and with the vessels shaking through an arc of 5 cm approximately 100 times per minute. Under these conditions there was no indication that the concentration of carbon dioxide was a limiting factor. The vessels were prepared with 1.0 ml of the bacterial suspension plus 0.5 ml of solution A (i.e.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  2.0 g, glycerol 20 g, sodium citrate 12.6 g, adjusted to pH 7 with HCl, and made up to 1 liter with distilled water) in the main space of the vessel, 0.5 ml of distilled water or a solution of ammonium chloride in the onset, and 0.3 ml of 10 per cent potassium hydroxide in the inset with filter paper.

*The determination of ammonia.* For this analysis the bacteria were separated from the suspending medium by filtration through fritted glass filters (pyrex, no. 36060, 15 UF) under reduced pressure. The filtrate was collected in 1 ml of 50 per cent (by volume) sulfuric acid.

The ammonia in the filtrate was determined by a procedure essentially the same as that described by Peters and Van Slyke (1932) in connection with the determination of urea in urine. To the acid filtrate was added distilled water to a volume of 10 ml and then 5 ml of 5 N KOH. Air, after passage through 5 per cent  $\text{H}_2\text{SO}_4$ , was drawn through the alkaline mixture and thence through 15 ml of 0.02N HCl, the ammonia being trapped in the latter. The total ammonia thus collected was estimated colorimetrically using a Cenco-Sheard-Sanford photometer, following the procedure outlined by Snell and Snell (1936), and using Jackson's modification of Nessler's reagent. This procedure can be used provided the quantity of ammonia present is not over about  $12 \times 10^{-2}$  mg. It is reproducible to within about  $0.25 \times 10^{-2}$  mg of ammonia in the sample filtered.

*Determination of total (Kjeldahl) nitrogen.* The contents of the respirometer vessel were washed into 1 ml of the digestion mixture (1 part saturated  $\text{K}_2\text{SO}_4$ , 1 part concentrated  $\text{H}_2\text{SO}_4$ , and a small amount of selenium powder; cf. Snell and Snell, 1936) in a pyrex test tube. Two glass beads were added and a glass bulb was placed on top. The tube was heated vigorously over a microburner until the water had been boiled off and the contents of the tube had begun to fume, the flame was reduced, and the mixture was allowed to boil gently until it was well charred. When charring had taken place, the tube was cooled for about 30 seconds, and a few drops of 30 per cent  $\text{H}_2\text{O}_2$  were dropped on the charred material. The mixture usually decolorized at once. It was then reheated, decolorized again if necessary, and finally boiled until it had remained clear for several minutes. This was taken as the end point of the digestion. The nitrogen then present as ammonium sulfate was determined exactly as described above for ammonium chloride.

## EXPERIMENTAL RESULTS

In order to establish the actual relationship between the uptake of ammonia and the rate of oxygen consumption, both processes were studied simultaneously. The experiments were conducted as follows: The respirometer vessels were prepared with the bacterial suspension and solution A in the main part of the vessel and with an amount of ammonium chloride (0.19 mg) which would sustain growth for only a few hours in the onset. After being shaken 1 hour in the constant temperature bath with the ammonium chloride in the onset, the bacteria

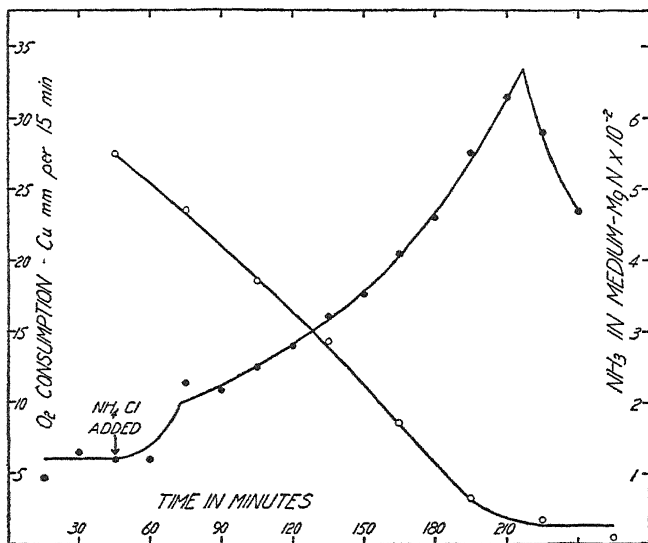


FIG. 1. A typical experiment showing as a function of time:  
 (1) the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens* (dots) and  
 (2) the quantity of ammonia present in the medium (circles).

Each point is the average result in two identical vessels. The ammonium chloride was added to the organisms from the onsets of the vessels at the point indicated.

reached a "resting" state, and the rate of oxygen consumption was comparatively steady although decreasing very gradually with time.<sup>1</sup> The ammonium chloride was then added to the bacteria. Measurements of the rate of oxygen consumption were continued, and at intervals the contents of the vessels were analyzed for ammonia, one of the vessels being removed for this purpose immediately following the addition of the ammonium chloride, and others every few minutes thereafter.

The results of a typical experiment are shown in figure 1 in which the solid circles indicate the rate at which oxygen was taken up in the respirometer vessels, whereas the open circles indicate the ammonia remaining in the medium. Before

<sup>1</sup> Any nutritive materials washed off the culture slants with the organisms were apparently in such low concentrations as to be completely metabolized during this initial hour in the respirometer.

the addition of ammonia, the rate of oxygen consumption is relatively constant and there is, of course, no growth. Upon adding ammonia, however, the medium becomes one which will support growth—it is, in fact, the one on which the organism was being maintained. At this point the rate of oxygen consumption rises quite abruptly and after some 20 to 30 minutes reaches a value which is nearly double the initial value. There then ensues a period during which the logarithm of the rate of oxygen consumption is a linear function of time. The curve drawn through the observed points in figure 1 during this phase of the experiment was obtained by calculation presuming that the logarithm of the rate is a linear function of time. It is clearly a good representation of the data. It undoubtedly represents the gradual increase in the quantity of bacterial protoplasm in the respirometer vessel, as others have noted (Greig and Hoogerheide, 1941; Hershey and Bronfenbrenner, 1938). From it the time for the bacterial mass to double, that is, for the logarithm of the rate of oxygen consumption to increase by the logarithm of 2, may be determined. This averaged 72 minutes (standard deviation,<sup>2</sup> 6 minutes) in 10 experiments.

It will be noted in the figure that the amount of ammonia present in the suspending medium decreases steadily throughout the experiment. It does so, of course, because it is taken up by the cells for elaboration into new protoplasm. The curve describing the utilization of the ammonia actually, therefore, represents the time course of the formation of new protoplasm. It is in fact a "growth curve."

As the concentration of ammonia approaches zero, the rate of oxygen consumption quite suddenly falls, just as has been described for *E. coli* (Armstrong and Fisher, 1947), to a relatively steady value. The latter is illustrated in the experiments of longer duration which are shown in figure 4 and which are to be discussed in detail below. In 9 experiments this resting rate was on the average 56.6 per cent of the maximum rate seen in the respirometer (standard deviation, 3.6 per cent). This steady (strictly, slowly declining) rate represents the resting rate which is characteristic of the amount of bacterial protoplasm now present in the respirometer vessel. Since the ammonia has been exhausted, it is evident that no appreciable uptake of ammonia can occur after the rate of oxygen consumption starts to decrease. It follows, then, that these organisms consume oxygen at either of two different rates, just as *E. coli* does, depending upon whether or not assimilation of ammonia is occurring.

This conclusion arises again when the rate of oxygen consumption and the rate of ammonia utilization are compared. As noted above, there is a rapid rise in the rate of oxygen consumption when the ammonia is first added. The rate of ammonia utilization, however, does not show any evidence of a similar initial spurt. It seems again, therefore, that in order to grow under these conditions the cells present must consume oxygen at a rate above that characteristic of a resting phase.

<sup>2</sup> The standard deviation was taken as  $\frac{(x - \bar{x})^2}{N - 1}$  where  $x$  is the result of one experiment,  $\bar{x}$  is the mean of the results, and  $N$  is the number of experiments.



It should perhaps be pointed out that it is not possible from the data available to estimate the difference between the activity and resting rates at the beginning of nitrogen assimilation. This results, firstly, from the lack of information about the existence of an initial lag period in the growth curve and, secondly, from the fact that the chemical systems involved here have considerable inertia, as indicated by the observation that, following the exhaustion of the nitrogen source, the rate of oxygen consumption does not decrease instantaneously.

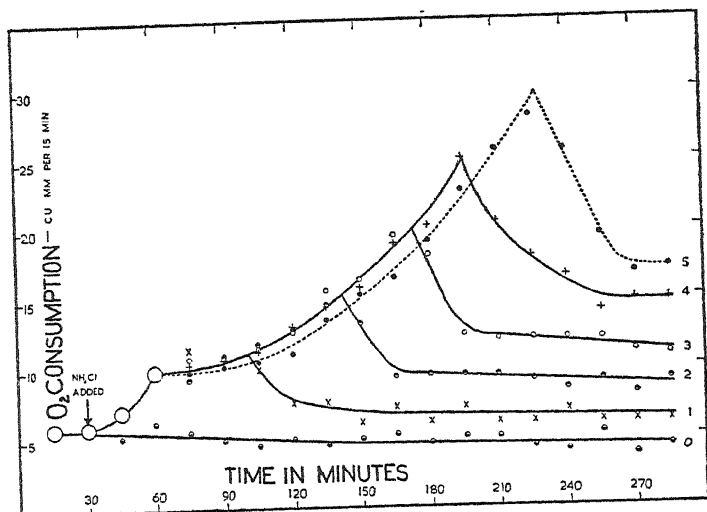


FIG. 2. The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens*. Each point is the average result in two identical vessels. As in figure 1 ammonium chloride was added to the organisms from the onset of the vessel at the point indicated. The numbers appearing at the right-hand end of each curve give, in hundredths of milligrams, the actual quantities of nitrogen added as ammonium chloride to the several vessels.

During the first hour the rates of oxygen consumption in the various vessels are essentially identical. Observations during this interval have, therefore, been indicated in the figure by single circles which have been made large enough to encompass all of the observations made at each time. In this particular experiment, although it was not usually so, the data for the highest concentration of ammonium chloride differed slightly from the curve describing the remainder of the points.\* To avoid confusion, therefore, the trend of these points is indicated by dashes.

To provide further information about the changes in the rate of oxygen consumption when ammonia is added or exhausted, the consequences of adding different quantities of ammonia were studied. These experiments were made by placing aliquots of bacterial suspension in each of several respirometer vessels, in the onsets of which different amounts of ammonium chloride were placed. As in the experiment described in figure 1, the resting rate of oxygen consumption was determined, and then the ammonia was tipped into all the vessels. Typical observations of the rate of oxygen consumption in an experiment of this kind are given in figure 2.

\* This would result if, by accident, fewer bacteria had been placed initially in one of these vessels.

In every case, when the ammonium chloride is added there is an initial rapid increase in the rate of oxygen consumption. This is followed by the gradual logarithmic increase already described. After the ammonia is exhausted, the rate of oxygen consumption falls to the lower resting rate.

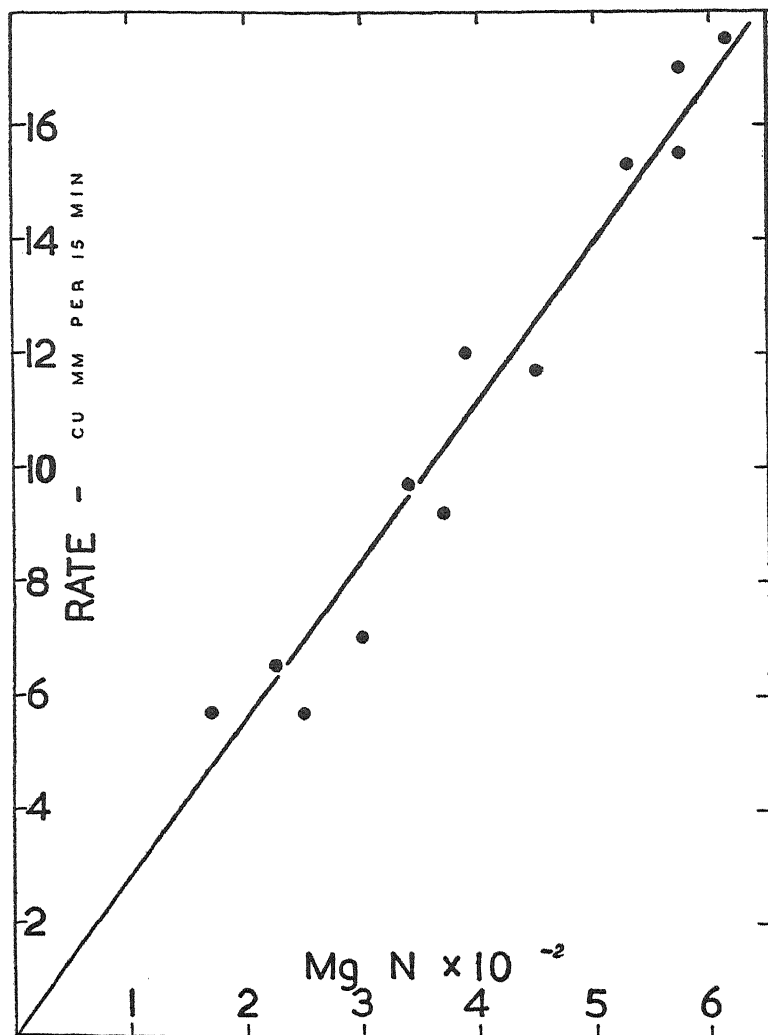


FIG. 3. A typical experiment in which the rate at which oxygen disappears from a respirometer vessel containing resting cells is shown as a function of the quantity of bacterial nitrogen present in the vessel. Each point is the result for one vessel.

It will be noted that the only significant differences between the several curves in figure 2 are the durations of the logarithmic phase and the absolute levels to which the rates fall after exhaustion of the ammonia. As might be expected, the logarithmic phase lasts longer, and the final resting level attained is higher, the

greater the amount of ammonia added. It was found that the resting rates were a constant percentage of the maximum rates reached, the percentage being independent, therefore, of the amount of growth which had taken place. As noted above, the resting levels averaged approximately 56 per cent of the peak rate.

At the termination of the experiments illustrated in figure 2, the contents of the vessels were analyzed for nitrogen. Determinations of the quantity of ammonia and total nitrogen in the suspending medium alone indicated that at this time all of the nitrogen present was in the cells. The analysis on the entire contents of the respirometer vessels thus measures the bacterial nitrogen present at this time. The latter can also, of course, be obtained by adding to the nitrogen present in the original aliquot of bacterial suspension, with which the experiment was begun, the amount of ammonia tipped into the vessel to initiate growth. In any case it is possible to compare the resting rates observed, after assimilation has ceased, with the amount of nitrogen present in the bacteria. This has been done in figure 3, and it is apparent there that the rate at which oxygen disappears in a respirometer vessel, containing resting cells, is directly proportional to the quantity of bacterial nitrogen which is present. It is to be noted that this is true even for the initial aliquots of bacteria, i.e., before any growth occurs in the respirometers. Moreover, the line in figure 3 passes through the origin, indicating that the nitrogen content is an absolute measure of the rate of oxygen consumption (cf. Hershey, 1939; Burris and Wilson, 1940). It is quite definite, therefore, that the several different resting rates recorded in figure 2 indicate the presence of different quantities of bacterial protoplasm. It may be calculated from the data in figure 3 that, on the average, these bacteria consumed oxygen at the rate of  $1.12 \times 10^3$  cu mm per hour per mg of nitrogen when suspended in solution A.

It is now evident that for any particular rate of oxygen uptake along the logarithmic part of the curve in figure 1, there is a corresponding lower resting rate to which the rate at which oxygen is disappearing would fall if the ammonia were suddenly removed. This lower rate was shown above to be determined solely by the amount of bacterial protoplasm present. Since it forms a constant percentage (approximately 56 per cent) of the activity rate, it is possible to plot on a graph, such as that in figure 1, a line which shows the time course of the resting rate following the addition of ammonia to the cells. During the period of logarithmic growth the resting rate is 56 per cent of the activity rate. To obtain the resting rate during the initial rapid rise in the rate of oxygen consumption, the curve describing the time course of the resting rate during logarithmic growth may be extrapolated backwards. Similarly the resting rate during the fall in the rate of oxygen consumption, following exhaustion of the ammonia, may be obtained by extrapolating backwards the nearly horizontal straight line which at the termination of the experiment describes the resting rate.

A calculated line giving the time course of the resting rate has been plotted along with a set of experimentally determined rates in figure 4. It is evident that the area enclosed by the lines describing, respectively, the observed rate of oxygen consumption and that indicating the time course of the resting rate rep-

resents the volume of oxygen consumed by the growing cells in excess of that required by resting cells. It is an accompaniment of the growth process. More specifically, it is the amount of oxygen consumed during the assimilation of a known quantity of nitrogen in the form of ammonia. The number of oxygen atoms consumed during the assimilation of each nitrogen atom given in the form of ammonia may, therefore, be calculated. The average value found in 10 experiments was 2.19, the standard deviation of the individual values about this mean being 0.14. This value was observed to be independent of the quantity of ammonia assimilated for quantities varying from 0.012 to 0.06 mg.

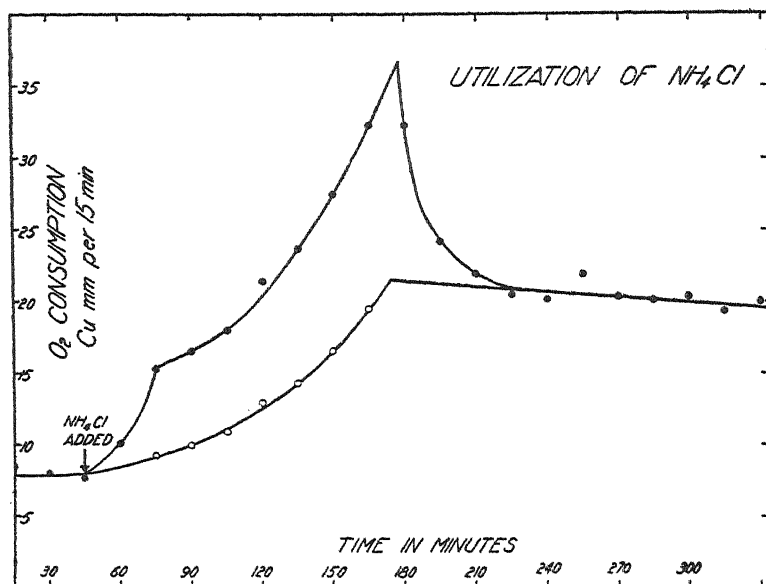


FIG. 4. The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens* (dots). Each point is the average of three identical vessels. Ammonium chloride was added at the point indicated. The calculated time course of the resting rate is indicated by circles.

#### SUMMARY

The oxygen consumption of the bacterium *Serratia marcescens* was studied in both growing and resting cells, and the rate of oxygen consumption per milligram of bacterial nitrogen was found to be higher when the assimilation of ammonia was taking place.

The extra oxygen used during the assimilation of the ammonia was determined. It was found that 2.2 oxygen atoms were taken up for each nitrogen atom assimilated.

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# FURTHER STUDIES ON THE IMMUNIZATION OF RABBITS TO TOXIGENIC *CORYNEBACTERIUM DIPHTHERIAE* BY INJECTIONS OF NONTOXIGENIC *DIPHTHERIA BACILLI*<sup>1</sup>

MARTIN FROBISHER, JR., AND ELAINE L. UPDYKE

*Department of Bacteriology, The Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland*

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Frobisher and Parsons (1943) reported that rabbits injected with broth cultures of living avirulent (nontoxigenic) *Corynebacterium diphtheriae* developed significant resistance to subsequent injections of living cultures of virulent (toxigenic) *C. diphtheriae*.

Their experiments involved 21 immunized and 11 control animals. These were tested with a challenge dose that was fatal to all of the 11 control rabbits, which died on an average of 3.6 days after the dose was administered.<sup>2</sup> Of the immunized animals 4 survived, the other 17 dying after an average of 7 days. In all, 48 per cent manifested some degree of resistance, including the 4 survivors. Resistance was ascribed to mobilization of cutaneous defenses, which effected, not a neutralization of toxin by antitoxin, but toxin localization. Antitoxin was not present. Apparently resistance was related to a greatly enhanced tissue reactivity and was presumably engendered by somatic antigens of the bacilli against a heterologous antigen—the exotoxin.

The present investigation was undertaken to extend and verify these observations and to collect further information regarding the phenomena observed.

## MATERIALS AND METHODS

*Infusion broth and agar.* These were prepared with veal or pork, according to the methods outlined in the *Manual of Methods* of the Society of American Bacteriologists, with the following modifications: (1) Neopeptone (Difco) was used in 1 per cent concentration, and (2) the meat infusion was heated to 80 C before pressing out the juice.

*Synthetic medium.* This was used in experiments to study the role of thiamine in the effectiveness of the antigens. The formula is given in the description of the experiments in which it was used.

*Cultures.* The avirulent strains of corynebacteria (cd107b and My654a) used as antigens were the same as those used by Frobisher and Parsons (1943), and tests for avirulence and atoxigenicity were not repeated. The virulent strain (EHD70) used for challenge doses was also the strain used by these workers. Broth cultures, 48 hours old, were used for both immunizing and challenge doses.

*Inoculations.* Several immunization programs were conducted with variations

<sup>1</sup> This study was aided by a grant from The Rockefeller Foundation.

<sup>2</sup> In later experiments using numerous controls no control animal has survived the same challenge dose.

in the route of inoculation, the total amount of antigen administered, the intervals between the injections of antigen, and the numbers of antigenic stimuli. However, within the limits employed<sup>3</sup> these variations appeared to have little or no effect on the degree of resistance of the rabbits to the subsequent challenge dose of virulent *C. diphtheriae*. The most commonly employed procedure for immunization consisted of ten 1-ml doses at intervals of 3 to 4 days. For the subcutaneous and intracutaneous inoculations the animals' backs were prepared by shaving with electric clippers.

The challenge doses were the same throughout the study. They consisted of 0.2 ml of a 48-hour broth culture of virulent *C. diphtheriae*, a dose which, with the strain employed, is uniformly fatal to normal rabbits. In general, it was administered 8 to 10 days after the last antigenic inoculation.

## EXPERIMENTS

### I. Veal-grown Antigens

At the time the work herein described was instituted, veal was being used routinely in this laboratory for preparing meat infusion media. Accordingly, veal infusion broth was used for the cultivation of the avirulent diphtheria bacilli with which the animals in this series of experiments were immunized. In all respects the procedures were made as nearly as possible like those previously used.

*Experiment 1a.* Ten rabbits were immunized with veal-grown antigen, 8 for a period of 4 weeks and 2 for 8 weeks. Following the challenge dose of virulent *C. diphtheriae*, all the animals died—7 within 24 to 48 hours, 1 on the third day, 1 on the fourth day, and 1 on the sixth day. All controls died at about the same rate. Because of the complete absence of resistance in the test animals, the experiment was repeated, only the period of immunization varying.

*Experiment 1b.* Eleven rabbits were immunized with veal-grown antigen, the immunization period being 5 weeks. Following the challenge dose, all of the animals died—5 within 2 days, 3 on the third day, and 3 on the fourth day.

The results of these two experiments were in surprising contrast to those previously obtained, i.e., the animals developed no resistance to virulent *C. diphtheriae*. The average survival time of the total of 21 test rabbits was 2.6 days and that of all 12 control (nonimmunized) rabbits was 2.7 days (table 1).

These two failures led to a careful review of the original work and revealed that the avirulent organisms with which the first animals had been immunized had been cultured in pork rather than veal infusion broth. This was because at the time of the original experiments pork was more readily available than veal. Immunizations were therefore repeated (exp. 2, 3, 4; 5a, and 5b) using cultures in pork infusion broth.

*Experiment 2.* Immunization with pork-grown antigen was started in 6 rabbits, but 3 died of nonspecific causes during the immunization period of 5

<sup>3</sup> Dosages varied from 7 ml of antigen given in 5 doses during 1 month to 46 ml of antigen given in 47 doses during 7 months.



weeks. Therefore, only 3 animals received the challenge dose of virulent *C. diphtheriae*. Of these 3, 2 survived and 1 died, but death did not occur until the seventh day. All 10 control animals died within 2 to 5 days.

*Experiment 3.* Six rabbits were immunized with pork-grown antigen over a period of 5 weeks. Following the challenge dose 2 animals survived, 2 died on the fifth day, 1 on the fourth day, and 1 on the third day.

TABLE 1

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent, veal-grown antigen*

EXPERIMENT NUMBER	PERIOD OF IMMUNIZATION	RABBITS	SURVIVAL TIME	AVERAGE SURVIVAL TIME
	<i>weeks</i>		<i>days</i>	<i>days</i>
1a	4	8	2-6	
	8	2	2	
1b	5	11	2-4	
Totals		21	2-6	2.6
Controls		12	2-5	2.7

TABLE 2

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent, fresh pork-grown antigen*

EXPERIMENT NUMBER	PERIOD OF IMMUNIZATION	RABBITS	SURVIVORS	LIFETIME OF NONSURVIVORS	AVERAGE LIFETIME OF NONSURVIVORS*
	<i>weeks</i>			<i>days</i>	<i>days</i>
2	5	3	2	7	7.0
3	5	6	2	3-5	4.2
4	5	5	2	5-8	6.0
5a	14	5	1	3-6	4.5
5b	29	3	1	5-11	8.0
Totals		22	8	3-11	5.4
Controls		10	0	2-5	3.7

\* Blood taken just before inoculation of virulent organisms was tested for antitoxin. Tests for 0.01 unit per ml were negative.

*Experiment 4.* Five rabbits were immunized with pork-grown antigen over a period of 5 weeks. Following the challenge dose 2 animals survived, 1 died on the eighth day, and 2 on the fifth day.

*Experiment 5a and 5b.* Eight rabbits were immunized with pork-grown antigen, 5 for a period of 14 weeks and 3 for 29 weeks. Of the first group of 5, following the challenge dose, 1 survived, and 1 died on each of the sixth, fifth, fourth, and third days. Of the 3 subjected to the longer immunization period, 1 survived, 1 died on the eleventh day, and 1 on the fifth day.

As shown in table 2, it is evident that the use of antigens cultivated in pork media afforded definite protection against virulent diphtheria bacilli. Of 22 rabbits immunized, 8 (36 per cent) survived the challenge dose. Others gave

evidence of increased resistance as evidenced by the 5.4-day average survival time of the 14 remaining rabbits. All of the 10 control rabbits died within 5 days or less.

A probable confirmation of the importance of *fresh* pork in the preparation of these antigens was later obtained inadvertently. Because of severe wartime shortages of meat fresh pork became unavailable. A preparation called, commercially, "pork-sausage," and probably consisting largely of corn meal and other nonporcine material, was used in cultivating antigens for one immunization experiment involving 12 rabbits. The results (table 3) were like those obtained with veal-grown antigens. At most only slight resistance was produced in the test animals. The average survival time was only 3.4 days as compared with 2 days for the controls. If the sausage contained fresh pork, which seems very unlikely, it must have been present in very small amounts, and its properties must have been modified by the spices and other materials mixed with it and by the processing to which it had been subjected.

TABLE 3

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with "pork-sausage" infusion broth*

PERIOD OF IMMUNIZATION	RABBITS	SURVIVAL TIME	SURVIVAL TIME AVERAGE
<i>weeks</i>		<i>days</i>	<i>days</i>
5	12	2-6	3.4
Control	1	2	2*

\* Compare also controls in tables 1, 2, 4, and 5.

## II. Reactions to the Challenge Dose

The local reaction to the challenge dose in most of the animals immunized with organisms grown in a fresh pork base (not "pork-sausage") medium was characteristic. An area of very marked edema, 6 to 12 cm, often more, in diameter, developed within 24 to 48 hours. Sometimes the whole flank of the animal was involved. This was gradually absorbed over a period of several days, and a corresponding but somewhat smaller area of necrosis developed. In contrast to these edematous reactions, the control animals, as well as most of the animals injected with organisms grown in media other than fresh pork infusion (including "pork-sausage"), developed much smaller lesions with little or no edema and much less extensive necrosis. Apparently resistance was closely related to the extent of the skin reaction.

As originally described, the resistant animals showed little or no evidence of general intoxication at any time, whether or not they survived, until a few hours before death if they died after several days. The controls and nonresistant (veal and "pork-sausage") animals were obviously ill within 24 to 36 hours after administration of the challenge dose. Evidently toxin was absorbed rapidly from the local lesion in the control and nonresistant animals but was held *in situ* in the resistant animals.

### III. Tests for Antitoxin

In order to have some confirmation of the observation that the survival of animals in these experiments is not dependent on the development in them of antitoxin, some of the test animals in this series were bled before receiving the challenge dose. The serum of 6 of the 8 animals surviving the challenge dose was examined and in each instance was found to contain less than 0.01 unit per ml. The sera were not assayed at lower levels.

### IV. Effect of Thiamine

From the results described above it was inferred that fresh pork contains some factor which is of critical significance in the antigenicity of avirulent diphtheria bacilli in regard to virulent diphtheria bacilli. Data on the amino acid and vitamin content of veal and pork were obtained from the American Meat Institute. According to these data an important difference between pork and veal is in the thiamine content, which is decidedly greater in pork.

Further experiments (exp. 6, 7, 8, 9, and 10) were conducted to verify the earlier results with veal-grown antigens and to determine whether or not thiamine had any influence on the phenomenon under study. The thiamine effect was studied with thiamine-enriched<sup>4</sup> veal infusion medium and with a synthetic medium developed in this laboratory and based on the method of Pappenheimer *et al.*<sup>5</sup> Efforts were made to use the media of Uschinsky (1893) and of Hadley

<sup>4</sup> One mg per cent thiamine chloride added before sterilization.

<sup>5</sup> Sodium lactate.....	6 ml	Valine.....	1 g
Glucose.....	1 g	Leucine.....	500 mg
MgSO <sub>4</sub> .....	1 g	Methionine.....	200 mg
K <sub>2</sub> HPO <sub>4</sub> .....	4 g	Tyrosine.....	100 mg
NaCl.....	6 g	Pimelic acid.....	10 mg
Tryptophane.....	200 mg	Beta-alanine.....	10 mg
Cysteine hydrochloride.....	200 mg	CuSO <sub>4</sub> .....	10 mg
Glycine.....	200 mg	H <sub>2</sub> O (dist.).....	1,000 ml
Glutamic acid.....	2 g		

Heat to dissolve.

Adjust with N/1 NaOH to pH 7.8 or 8.0.

Boil vigorously for 5 min.

Add distilled water to restore volume.

Cool to room temperature.

Filter through a good grade of filter paper.

Dispense and sterilize in the autoclave (15 lb, 20 min).

To each 100 ml of this base add aseptically 0.2 ml of vitamin solution 1 or 2.

#### Vitamin Solution 1

Ascorbic acid.....	100 mg
Niacinamide.....	40 mg
Riboflavin.....	8 ml
(100 mg % in H <sub>2</sub> O)	
Pyridoxine.....	1 ml
(100 mg % in H <sub>2</sub> O)	
Calcium pantothenate.....	2 ml
(100 mg % in H <sub>2</sub> O)	
H <sub>2</sub> O.....	89 ml

Sterilize by Seitz filtration.

#### Vitamin Solution 2

To Vitamin Solution 1 add:	
Thiamine chloride.....	350 mg
Sterilize by Seitz filtration.	

(1907) and the formula of Pappenheimer, Mueller, and Cohen (1931). However, the strains of *C. diphtheriae* in use failed to grow in serial subcultures in any of these three media. Therefore, the synthetic medium described below was developed. This maintained the organisms in indefinite serial transfer.

*Experiment 6.* Six rabbits were immunized with veal-grown antigen for a period of 5 weeks. Following the challenge dose of virulent *C. diphtheriae* all the animals died, 1 within 2 days, 1 on the third day, 3 on the fourth day, and 1 on the fifth day.

*Experiment 7.* Ten rabbits were immunized with veal-grown antigen for a period of 5 weeks. Following the challenge dose all the animals died, 6 on the second day and 4 on the third day.

*Experiment 8.* Ten rabbits were immunized with thiamine-enriched veal-grown antigen for a period of 5 weeks. Following the challenge dose all the animals died within 2 days.

TABLE 4

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with veal infusion media with and without added thiamine*

EXPERIMENT NUMBER	PERIOD OF IMMUNIZATION	RABBITS	SURVIVAL TIME	AVERAGE SURVIVAL TIME
	<i>weeks</i>		<i>days</i>	<i>days</i>
6*	5	6	2-5	3.6
7*	5	10	2-3	2.4
8†	5	10	2	2
Controls		4	2-4	2.8

\* Veal infusion medium without added thiamine.

† Veal infusion medium with added thiamine.

*Experiment 9.* Four rabbits were immunized with antigen grown in synthetic medium without thiamine for a period of 5 weeks. Following the challenge dose all the animals died, 1 on the fourth day, 2 on the fifth day, and 1 on the seventh day.

*Experiment 10.* Three rabbits were immunized in the usual manner, using antigen cultivated in synthetic medium with thiamine. Following the challenge dose all the animals died, 1 on the third day and 2 on the fifth day.

The results of these experiments, summarized in tables 4 and 5, corroborated the earlier evidence of the relative inefficacy of veal-grown antigens, since the average survival time of 46 animals receiving veal-grown antigens (exp. 1a, 1b, 6, 7, and 8) was 2.6 days and that of 4 controls 2.6 days. These animals all died, whereas 36 per cent of the animals receiving pork-grown antigens survived and those dying lived longer (avg 5.4 days) than the controls (avg 2.6 days).

These experiments also served to demonstrate that thiamine is apparently not the factor in pork which determines the protective antigenicity of avirulent *C. diphtheriae*. The average survival time of 41 rabbits (exp. 1a, 1b, 6, 7, and 9) receiving low-thiamine antigen (veal and synthetic media) was about 3.5 days;

that of 13 rabbits (exp. 8, 10) receiving high-thiamine antigen (veal and synthetic media) was 2.5 days. All these rabbits died.

## DISCUSSION

A review of all the experiments reveals that only those rabbits that were immunized with avirulent organisms cultivated in fresh pork infusion media developed any definite resistance to infection with virulent diphtheria bacilli.

TABLE 5

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with synthetic media with and without added thiamine*

EXPERIMENT NUMBER	PERIOD OF IMMUNIZATION	RABBITS	SURVIVAL TIME	AVERAGE SURVIVAL TIME
	<i>weeks</i>		<i>days</i>	<i>days</i>
9*	5	4	4-7	5.2
10†	5	3	3-5	4.3
Controls		4	2	2

\* Synthetic medium without thiamine.

† Synthetic medium with thiamine added.

TABLE 6

*Summary table: Reactions of rabbits to virulent challenge dose following immunization with avirulent antigen prepared in a variety of media*

MEDIUM USED FOR CULTIVATING IMMUNIZING ORGANISMS	RABBITS	SURVIVORS	PERCENTAGE OF SURVIVALS	AVERAGE LIFETIME OF NONSURVIVORS	MAXIMUM LIFETIME OF NONSURVIVORS
				<i>days</i>	<i>days</i>
Medium containing a* fresh pork	21	4	19	7.0	13
b†	22	8	36	5.3	11
Totals.....	43	12	28	6.0 ( $\pm 0.5$ )	13
Medium not containing fresh pork	66	0	0	3.2	7
Controls	31	0	0	2.9	5

\* Data from Frobisher and Parsons (1943).

† Data from present report.

A summary table (table 6), in which data on animals immunized with antigens grown in fresh pork media are contrasted with data on animals immunized with antigens grown in veal and synthetic media and with data on the control animals, brings into sharp relief the difference in the protection afforded. It seems obvious that fresh pork contains some factor which determined the efficacy of avirulent *C. diphtheriae* as an antigen inducing resistance in rabbits against virulent *C. diphtheriae*.

An effort to learn the nature of this factor served merely to demonstrate that thiamine is not the responsible agent.

These studies have amply corroborated the earlier finding (Frobisher and Parsons, 1943) that under proper experimental conditions rabbits that receive repeated doses of cultures of avirulent diphtheria bacilli develop a resistance to, and in many cases survive, doses of virulent diphtheria bacilli that are invariably fatal to normal rabbits. Two important additional facts have also been established: (1) as between the media used here, a fresh pork base medium is essential to antigenic effect; (2) thiamine is not per se responsible for the antigenic effect.

The implications of these findings are fairly obvious with respect to media used in the preparation of antigens heretofore regarded as of little efficacy, such as dysentery and cholera vaccine, etc., and the improvement of bacterial antigens already in use, such as typhoid and pertussis. The antigens might be made more effective by the inclusion in their culture media of some essential factor such as the yet unknown "pork factor" described here.

The mechanism of the protection afforded by the avirulent diphtheria bacilli is noteworthy but not understood. Allergy apparently is not significant, for there is no enhanced skin reactivity to the *homologous* somatic antigen of the avirulent bacilli, but only to the heterologous antigen—the exotoxin of the virulent organisms. That resistance and survival are not due to the presence of antitoxin in the blood stream was pointed out by Frobisher and Parsons (1943) and was again demonstrated in these studies. Judging by the appearance of the local reaction and the relatively "bright" appearance of the test rabbits following the challenge dose, it would seem that there is some local tissue reaction which binds the toxin, delaying its general absorption or, in the case of the survivors, entirely preventing absorption by holding the toxin *in situ* until the animal has built up its own antitoxic (and possibly antibacterial) antibodies to combat the infection.

In a general sense this is reminiscent of the observations by Abernethy and Francis (1937) that "some factor or change occurring in the serum in response to bacterial pneumococcal infection is capable of being mobilized in tissues and thereby reacting locally with the C substance" and that "the state of reactivity of the tissue cells is also essential for cutaneous response to C."

Whatever the nature of the phenomenon, it is obvious that some protection is afforded. In view of this fact, as well as of the mounting evidence that what is generally considered an adequate program of toxoid immunization is not always sufficient to prevent diphtheria (Eller and Frobisher, 1945; Turner, 1942), it seems permissible to suggest again that consideration be given to the idea that the immunizing agents used to protect children against diphtheria should contain properly cultivated bacterial antigens as well as antigens to stimulate anti-exotoxin.

#### SUMMARY AND CONCLUSIONS

Eighty-eight rabbits were repeatedly inoculated with living cultures of avirulent *Corynebacterium diphtheriae*. Twenty-two of the 88 animals received organisms which had been cultivated in a pork infusion medium. Of these 22,

8 (36 per cent) survived a subsequent challenge dose of virulent *C. diphtheriae* which was uniformly fatal to nonimmunized animals. The other 14 animals in this group of 22 survived an average time of 5.3 days as contrasted to the 2.9-day average survival time of 31 control (nonimmunized) animals.

This is in contrast with 66 rabbits which received inoculations of avirulent *C. diphtheriae* cultivated on media not containing fresh pork. Of these 66 animals, none survived the challenge dose of virulent *C. diphtheriae*, and their average survival time of about 3 days was essentially the same as that (2.9 days) of the 31 control animals. Thiamine was shown not to be the essential antigen-adjuvant in the pork. The implications of these results have been discussed briefly with respect to immunization procedures in general, and especially those against diphtheria.

Partial or complete protection against virulent diphtheria bacilli was engendered in rabbits by injecting into them living cultures of avirulent diphtheria bacilli which had been cultivated in a fresh pork base medium.

Avirulent *C. diphtheriae* cultivated in certain media not containing fresh pork were incapable of engendering any significant resistance against the virulent organisms.

Fresh pork contains some factor which is critical for the antigenicity of the avirulent diphtheria bacilli under the conditions of these experiments. This factor is apparently not thiamine.

The resistance of the immunized animals was not due to the presence of demonstrable antitoxin in the blood stream, and the mechanism of the protective action is not antitoxic. It appears to depend rather on a local binding action in subcutaneous tissues, where the unneutralized toxin causes extensive necrosis.

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# A STUDY OF BACTERIAL SYNERGISM WITH REFERENCE TO THE ETIOLOGY OF MALIGNANT DIPHThERIA<sup>1, 2</sup>

ELAINE L. UPDYKE AND MARTIN FROBISHER, JR.

*Department of Bacteriology, The Johns Hopkins School of Hygiene and Public Health,  
Baltimore, Maryland*

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Malignant, "bull-neck," hypertoxic, or grave diphtheria is differentiated from "ordinary" or milder diphtheria by the presence of marked cervical swelling (hence "bull-neck"), extreme toxemia, albuminuria, frequent development of neuropathies, and a high death rate in spite of early administration of large doses of antitoxin. Epidemics of diphtheria in which the malignant form predominated have been common in Europe, England, and elsewhere within the past two decades as reported by Anderson *et al.* (1931) and Deicher and Agulnik (1927), but during the same period have been relatively uncommon on this continent. Since 1931 only one such outbreak has been reported (Wheeler and Morton, 1942). However, in Baltimore, after several years of low morbidity and fatality, diphtheria began to increase in 1942 and, as in many other large cities in the United States and Europe during the war years, it attained high levels (Eller and Frobisher, 1945). During the first 6 months of 1944 there were in Baltimore 142 cases, 16 of which were designated as malignant. The malignant cases suffered a 44 per cent mortality (7 cases), whereas the total mortality was only 6 per cent (9 cases). In 1945 there were 352 cases reported and 18 deaths. A considerable number of these were malignant. The disease continued at a relatively high level of incidence and severity throughout 1946.

Since the description of *gravis* and *mitis* types of diphtheria bacilli by Anderson *et al.* (1931), malignant diphtheria has been widely believed to be due to the *gravis* type of diphtheria bacilli, although it has been repeatedly pointed out by Frobisher (1943) and others that the occurrence of this organism, at least that variety of it which is found in Baltimore and elsewhere in the United States, bears no constant relation whatever to malignant diphtheria. Continuous, systematic studies of the types of diphtheria bacilli found in cases of diphtheria in Baltimore since about 1932, including the numerous typical, fatal malignant cases noted above, have revealed during 16 years only 10 or 12 *gravis* strains, and these rarely in the malignant cases. The *mitis* or *mitis*-like form has predominated in cases, contacts, and carriers at all times according to Frobisher (1938, 1940, 1942). This has also been found true, as a general rule, throughout the United States. Obviously, then, malignant diphtheria in Baltimore and the United States during

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the period mentioned has depended on some other factor than the strains of *gravis* or *mitis* diphtheria bacilli found there.

The possibility that a factor in malignant diphtheria might be the synergistic action of other bacteria has long been a subject of debate and experimentation. Since the time of Roux and Yersin (1890) streptococci have most frequently been mentioned and investigated in this connection. Unfortunately, studies prior to 1903 did not take into account the variability in virulence and toxigenicity of cultures of *Corynebacterium diphtheriae*. Many workers did not use enough animals to give significance to their results. None of them could identify their strains of streptococci with accuracy since they were working before the introduction of the bile solubility test for differentiating streptococci and pneumococci, the use of the blood agar plate, the method of differentiating streptococci on the basis of the type of hemolysis on blood agar, and the precipitin method of grouping beta hemolytic streptococci.

More recent experimental studies on this problem have been conducted by Ramon and Djourichitch (1934). These authors concluded, in contrast with Roux and Yersin, that streptococci lower local tissue resistance to invasion by diphtheria bacilli but do not enhance the virulence of the latter. Certain objections to these conclusions will be cited later.

Dold (1927), Hopmann and Panhuysen (1931), Park and Williams (1933), and Stimson (1940) considered malignant diphtheria as due to combined infections. Goepf (1938) and Cushing (1943) list several organisms that they believe may be involved. These and most other authors stress the importance of streptococci. The different views with regard to the role of streptococci and other factors in malignant diphtheria have been summarized previously (Frobisher, 1943).

Our own interest in streptococci as related to malignant diphtheria was kindled by the isolation of hemolytic streptococci of Lancefield group B from several successive cases of malignant diphtheria in Baltimore. The diphtheria bacilli associated with these streptococci were all of the *mitis* type. Subsequent experiments with these organisms in animals inclined us to the view that such streptococci are probably of etiological significance in malignant diphtheria. Later studies of streptococci found in other cases of malignant diphtheria, however, failed to support this view, and left us unconvinced, one way or the other. The experiments described below were therefore undertaken to investigate further the problem of bacterial associations in this disease.

#### MATERIALS AND METHODS

*Cultural procedures.* The cultural methods and media used were of a common type routinely employed in this laboratory and have been described elsewhere (Frobisher, 1938).

*Bacteria.* Five strains of virulent *C. diphtheriae* (nos. C1 to C5) and two avirulent strains (C6 and C7) were used. These are described in table 1.

Five strains of beta type hemolytic streptococci isolated from the same patients as those yielding the correspondingly numbered virulent diphtheria strains

were also used. These are included in table 1, with notation of their Lancefield group.

These pairs of diphtheria bacilli and streptococcus cultures, each pair from the same patient, are called "homologous pairs" or "homologous strains" in this report.

One strain each of *Corynebacterium xerose* and *Corynebacterium pseudo-diphthericum* were isolated from normal persons.

*Other organisms.* Three strains of *Hemophilus influenzae*, type B, were obtained from the National Institute of Health.<sup>3</sup>

*Diphtheria toxin.* A well-ripened toxin (T106) produced by a Park Williams no. 8 strain of *C. diphtheriae*, and of tested potency, was used. All dilutions of the toxin were made with Moloney and Taylor diluent prepared according to the

TABLE 1

STRAIN NO.	TYPE	SOURCE	ASSOCIATED STREPTOCOCCI	
			Strain no.	Lancefield group
C1	mitis	malignant diphtheria	S1	B
C2	mitis	malignant diphtheria	S2	B
C3	mitis	malignant diphtheria	S3	A
C4	minimus	malignant diphtheria	S4	B
C5	gravis	moderately severe diphtheria	S5*	A
C6	gravis	healthy carrier		
	avirulent			
C7	gravis	healthy carrier		
	avirulent			

\* Streptococcus strain S5 came from a malignant case which was a contact of the C5 patient. Both cases yielded a gravis type of diphtheria bacillus.

formula in *Diagnostic Procedures and Reagents* of the American Public Health Association (1945).

*Animal experiments.* Basically all of these experiments were alike, consisting in the injection of diphtheria bacilli or diphtheria toxin mixed with various other bacteria or substances into test animals. The arrangement of controls was given particular attention, and each arrangement is described in detail in the appropriate place.

#### EXPERIMENTS WITH MICE

White mice were selected as experimental animals because of their relatively great resistance to *C. diphtheriae* and its toxin. This resistance was considered advantageous for two reasons: (1) Any synergistic reaction which might manifest itself would be the more obvious. (2) Larger doses of diphtheria bacilli could be inoculated alone, for control purposes, or in combination with other organisms, without using antitoxin to prevent early deaths of the mice from diphtheritic

<sup>3</sup> Courtesy of Dr. Margaret Pittman of the Biologics Control Laboratory, National Institute of Health.

intoxication. In most experiments sublethal doses of diphtheria bacilli and streptococci were injected together intraperitoneally into 6 mice in a group. The mixtures of organisms were made 15 minutes before injection.

The results were compared with controls made up of similar groups of mice each receiving a dose of each organism alone equal to the largest amount of that organism in any tested combination. The proportions of one organism to the other varied in different experiments. Time and numbers of deaths were the criteria of severity of infection. A maximum observation period of 14 days was set arbitrarily. In order to minimize variables, age and weight limitations for the mice were adopted for most of the tests. It was found most convenient to use mice 6 to 9 weeks old weighing 18 to 23 grams. Closer limits were impracticable because of wartime shortages of mice. Cultures in broth, 48 hours old, were used routinely. It is to be noted, particularly in these first experiments, that, if a given dose of culture was to be less than 0.1 ml., the culture was diluted with sterile infusion broth so that the amount to be injected was 0.1 ml.

The diphtheria bacilli and streptococci in these experiments were homologous pairs.

#### PRELIMINARY EXPERIMENTS

*Virulent C. diphtheriae and beta hemolytic streptococci.* A previously determined sublethal dose of each organism was inoculated into control groups of mice, and the same or smaller doses were combined for inoculation into the test groups. In most cases this meant that the total volume of fluid inoculated into the test animals was greater, by 0.2 ml, than that given any of the controls. This was at first considered a negligible difference.

Experiments of this type were conducted with the following homologous pairs of virulent *C. diphtheriae* and hemolytic streptococci (beta type): C1 and S1, C2 and S2, C3 and S3, C4 and S4, and C5 and S5. Protocols and results of two representative experiments are shown in table 2. In all instances the animals receiving a combination of virulent *C. diphtheriae* and beta hemolytic streptococci of Lancefield group B died in much greater numbers, or more rapidly, or both, than those receiving either organism alone. The tentative inference drawn and reported at this time (Updyke and Frobisher, 1944) was that the reactions were evidence of a synergistic action between the two organisms.

*Experiments with hemolytic streptococci (beta type) plus avirulent C. diphtheriae and nonspecific substances.* Later, experiments were conducted with three strains of hemolytic streptococci (S1, S2, S4) in various combinations with (1) living and killed cultures of two strains of avirulent *C. diphtheriae* (C6, C7); (2) sterile 10 per cent suspensions of animal charcoal and sterile 1 per cent suspensions of diatomaceous earth (pulverized Berkefeld filter) in infusion broth; and (3) sterile infusion broth. Typical results are shown in table 3.

In general, deaths occurred in greater numbers, in a shorter time, or both, in the animals receiving the combinations of living streptococci and other substances *irrespective of the nature of the added substance. Deaths were in nearly direct proportion to the volume of the inoculum.*

It was not determined how much of the enhanced effect of the streptococci may have been due to the nutrient factor resulting from greater volume of broth and how much to the mechanical factors of size of inoculum and amount of particulate matter. Whatever the explanation, it was evident that the results in the previously described preliminary experiments with virulent *C.*

TABLE 2

*Results of intraperitoneal inoculation into mice of virulent C. diphtheriae and beta type hemolytic streptococci combined*

RELATION OF CULTURES	ORGANISMS INOCULATED				MICE					
	Species	Strain no.	Type or group	Amount (ml)	No. inoculated	Cumulative no. dead (days)				Ratio of deaths to no. inoculated
						1	4	7	14	
Control*	<i>C. diphtheriae</i>	C1	mitis	0.2	6	0	0	0	0	0/6
Control*	<i>S. hemolyticus</i>	S1	B	0.2	6	0	0	1	1	1/6
Combined	<i>C. diphtheriae</i>	C1		0.2	6	2	4	6	6	6/6
	<i>S. hemolyticus</i>	S1		0.2						
Combined	<i>C. diphtheriae</i>	C1		0.2	6	1	4	6	6	6/6
	<i>S. hemolyticus</i>	S1		0.1						
Combined	<i>C. diphtheriae</i>	C1		0.1	6	0	1	2	4	4/6
	<i>S. hemolyticus</i>	S1		0.2						
Control*	<i>C. diphtheriae</i>	C2	mitis-like	0.3	6	0	0	0	0	0/6
Control*	<i>S. hemolyticus</i>	S2	B	0.1	6	0	0	0	0	0/6
Combined	<i>C. diphtheriae</i>	C2		0.3	6	4	4	5	6	6/6
	<i>S. hemolyticus</i>	S2		0.1						
Combined	<i>C. diphtheriae</i>	C2		0.3	6	4	5	5	5	5/6
	<i>S. hemolyticus</i>	S2		0.05†						
Combined	<i>C. diphtheriae</i>	C2		0.2	6	1	4	4	4	4/6
	<i>S. hemolyticus</i>	S2		0.1						

\* Culture alone.

† 0.1 ml of a 1:2 dilution.

*diphtheriae* were probably due to these factors rather than to a synergistic action between the diphtheria bacilli and streptococci.

Further experiments with virulent and avirulent *C. diphtheriae*, hemolytic streptococci (beta type), and nonspecific substances. In these experiments the combined action of homologous pairs of virulent diphtheria bacilli and streptococci was again studied, but in these tests there were included control groups of mice receiving each organism in combination with avirulent diphtheria bacilli (C6), inert particles, or infusion broth. An acute war-induced shortage of mice limited these fully controlled experiments to three: two with organisms C1 and

S1, and one with C2 and S2. In each experiment a total of 15 to 16 control and

TABLE 3

*Results of intraperitoneal inoculation in mice of beta type hemolytic streptococci combined with suspensions of living and killed avirulent C. diphtheriae, charcoal, diatomaceous earth, and sterile infusion broth*

RELATION OF MATERIALS	MATERIALS INOCULATED				MICE					
	Species and substances	Strain number	Type or group	Amount (ml)	No. inoc- ulated	Cumulative no. dead (days)				Ratio of deaths to no. inoculated
						1	4	7	14	
Control*	<i>C. diphtheriae</i>	C6	gravis	0.8	6	0	0	0	0	0/6
Control*	<i>S. hemolyticus</i>	S1	B	0.2	6	0	1	1	1	1/6
Combined	<i>C. diphtheriae</i>	C6	gravis	0.8	6	6	6	6	6	6/6
	<i>S. hemolyticus</i>	S1	B	0.2						
Combined	<i>C. diphtheriae</i>	C6	gravis	0.6	5	4	4	4	4	4/5
	<i>S. hemolyticus</i>	S1	B	0.2						
Control*	<i>C. diphtheriae</i>	C7	mitis	0.7	6	2	2	2	2	2/6
Control*	<i>C. diphtheriae</i>	C7	mitis†	0.7	6	0	0	0	0	0/6
Control*	<i>S. hemolyticus</i>	S4	B	0.025‡	6	0	5	5	5	5/6
Control*	<i>S. hemolyticus</i>	S4	B†	0.025‡	6	0	0	0	0	0/6
Combined	<i>C. diphtheriae</i>	C7	mitis	0.7	6	6	6	6	6	6/6
	<i>S. hemolyticus</i>	S4	B	0.025‡						
Combined	<i>C. diphtheriae</i>	C7	mitis†	0.7	6	6	6	6	6	6/6
	<i>S. hemolyticus</i>	S4	B	0.025‡						
Combined	<i>C. diphtheriae</i>	C7	mitis	0.7	6	0	0	0	1	1/6
	<i>S. hemolyticus</i>	S4	B†	0.025‡						
Control*	<i>S. hemolyticus</i>	S4	B	0.025‡	6	0	2	2	2	2/6
Combined	Charcoal 10%§			0.1	6	1	5	6	6	6/6
	<i>S. hemolyticus</i>	S4	B	0.025‡						
Combined	Berkefeld filter			0.2	6	0	4	4	4	4/6
	1%§									
	<i>S. hemolyticus</i>	S4	B	0.025‡						
Combined	Infusion broth			0.8	6	6	6	6	6	6/6
	<i>S. hemolyticus</i>	S4	B	0.025‡						

\* Culture alone.

† Killed: 56 C waterbath 75 min.

‡ 0.1 ml of a 1:4 dilution.

§ Suspended in infusion broth.

test groups of mice were inoculated within 2 to 3 hours with cultures from the same sources.

TABLE 4

Results of intraperitoneal inoculation in mice of *C. diphtheriae*, beta type hemolytic streptococci, and nonspecific agents, alone and in combination

RELATION OF MATERIALS	MATERIALS INOCULATED					MICE					
	Organisms or nonspecific substance	Strain no.	Type or group	Viru- lence	Amount (ml)	No. inocu- lated	Cumulative no. dead (days)				Ratio of deaths to no. inoculated
							1	4	7	14	
Control*	<i>C. diphtheriae</i>	C1	mitis	+	0.2	12	0	0	0	1	1/12
Control*	<i>C. diphtheriae</i>	C6	gravis	—	0.6	12	0	0	0	0	0/12
Control*	<i>S. hemolyticus</i>	S1	B		0.1	12	0	0	0	0	0/12
Combined	<i>C. diphtheriae</i> Infusion broth	C1	mitis	+	0.2 0.1	6	0	0	0	0	0/6
Combined	<i>C. diphtheriae</i> Charcoal 10%†	C1	mitis	+	0.2 0.1	12	0	0	0	0	0/12
Combined	<i>C. diphtheriae</i> Infusion broth	C1	mitis	+	0.2 0.6	12	0	0	0	0	0/12
Combined	<i>C. diphtheriae</i> Charcoal 10%†	C1	mitis	+	0.2 0.6	12	0	0	0	0	0/12
Combined	<i>C. diphtheriae</i> <i>C. diphtheriae</i>	C1 C6	mitis gravis	+	0.2 0.6	12	0	2	4	5	5/12
Combined	<i>C. diphtheriae</i> Infusion broth	C6	gravis	—	0.6 0.2	12	0	0	0	0	0/12
Combined	<i>C. diphtheriae</i> Charcoal 10%†	C6	gravis	—	0.6 0.2	12	0	0	0	0	0/12
Combined	<i>S. hemolyticus</i> Infusion broth	S1	B		0.1 0.2	12	0	1	1	2	2/12
Combined	<i>S. hemolyticus</i> Charcoal 10%†	S1	B		0.1 0.2	12	3	3	3	3	3/12
Combined	<i>S. hemolyticus</i> <i>C. diphtheriae</i>	S1 C1	B mitis		0.1 0.2	12	0	2	2	3	3/12
Combined	<i>S. hemolyticus</i> Infusion broth	S1	B		0.1 0.6	12	4	6	6	8	8/12
Combined	<i>S. hemolyticus</i> Charcoal 10%†	S1	B		0.1 0.6	12	10	12	12	12	12/12
Combined	<i>S. hemolyticus</i> <i>C. diphtheriae</i>	S1 C6	B gravis		0.1 0.6	12	7	9	11	11	11/12

\* Culture alone.

† Suspended in infusion broth.

The results obtained with both pairs of organisms (C1 + S1, C2 + S2) were closely parallel. Representative data are shown in a composite table of the two tests run with C1 and S1 (table 4). The combination of the streptococcus (S1) with equal amounts (0.2 ml) of virulent *C. diphtheriae* (C1), 10 per cent charcoal, or infusion broth resulted in a definite, though small, increase in deaths as compared with controls: 3, 3, and 2, respectively, among 12 animals. Only 1 of the *C. diphtheriae* and none of the streptococcus control animals died. Combination of streptococci with larger amounts (0.8 ml) of any agent, whether avirulent *C. diphtheriae*, 10 per cent charcoal suspension, or sterile infusion broth, resulted in a much more marked increase in mouse deaths: 11, 12, and 8, respectively, among groups of 12 animals.

In contrast with the above-described results obtained by combining various agents with streptococci, the combining of inert particles, infusion broth, and other agents with virulent and avirulent diphtheria bacilli did not alter the results; i.e., all the mice survived.<sup>4</sup>

#### DISCUSSION

The results of our first experiments with streptococci were thought to indicate a synergistic interaction between certain strains of diphtheria bacilli and beta hemolytic streptococci. In later experiments, however, similar results were obtained with three strains of streptococci in combination with nonspecific substances. It is probable, therefore, that all the results involving streptococci were a manifestation of the influence of volume of fluid, pabulum, or particulate matter on these organisms (or on host resistance) and that no synergism occurred in any of the tests. In contrast with streptococci, the strains of *C. diphtheriae* used in this study exhibited no enhancement of lethal effect as a result of similar combinations.

In view of these results and of the observations of Djamil (1934) regarding the importance of the quality of the suspending fluid in such experiments, the role of the streptococci in the reactions reported by Ramon and Djourichitch (1934), and by ourselves, is debatable. The latter workers first determined sublethal doses of saline suspensions of *C. diphtheriae* and then inoculated guinea pigs with such suspensions in combination with streptococcus cultures in broth or in filtrates of these. It seems probable that they would have obtained similar results with combinations of the streptococcal suspensions and sterile broth. Similar errors probably existed in many of the early investigations.

In conclusion of this discussion on the experiments with mice, it may be said that under these experimental conditions—

- (1) The lethality of some strains of beta hemolytic streptococci for mice is

<sup>4</sup> In one test, for an unexplained reason, 5 of 6 mice died after receiving the virulent-avirulent diphtheria bacilli combination (C1 + C6), but all 6 animals survived the corresponding inoculation in the parallel test with C2 and C6. Also, the virulent-avirulent diphtheria bacillus combination (C2 and C7) was not lethal to the mice. Unfortunately, no more mice were available for further study.



markedly affected by the amount of nutrient material, particulate matter, or both injected with them, and

(2) The increased lethal effect observed when *C. diphtheriae* and beta type hemolytic streptococci are combined can best be explained as due to the increased volume of nutrient material. In view of this, the increased lethality observed in mice in these experiments cannot be attributed to a true synergistic action between the two organisms.

#### EXPERIMENTS WITH RABBITS

It was necessary to discontinue the line of investigation just described because of a critical shortage of mice arising from greatly increased military demands. Since the supply of guinea pigs was also limited, the investigation was continued employing rabbits as experimental animals. Their use necessitated a considerable modification in technique because of the extreme susceptibility of rabbits to diphtheria infection and intoxication. Modifications were also made to eliminate error due to uncontrolled variations in the volume and nature of inoculum such as those which had proved to be so important in the experiments with mice. In addition, the study was expanded to include organisms other than streptococci.

In general, 3 rabbits were used in parallel for each test, all 3 receiving the same control and combined inocula. Inoculations were made intracutaneously in the dorsal skin of rabbits which had been shaved with electric clippers. The relative position of control and combined inoculations were the same for all three animals in a single test but were varied from one test to another to eliminate the possibility of differences in local tissue response. In all injections the volume of the inoculum was 0.2 ml. The quality of the fluid varied in accordance with the nutritive requirements of the organisms under study. Observations on the animals were continued until all reactions were subsiding (usually 2 to 4 days).

#### PRELIMINARY TESTS

*Virulent C. diphtheriae and hemolytic streptococci (beta type).* A few tests were carried out with living cultures of virulent *C. diphtheriae* and streptococci, Lancefield group B (C1 and S1), alone, in combination with each other, and in combination with 10 per cent animal charcoal in infusion broth. Four hours after the test injections the animals were given 1,000 units of diphtheria antitoxin intravenously in order to prevent early deaths from diphtheritic intoxications. Immediately after the administration of antitoxin, duplicate inoculations in adjacent sites were made of all the test materials to determine whether the combined infections were antitoxin-refractory.

The following results were obtained in these tests:

(1) The areas of erythema and necrosis produced by the combinations of diphtheria bacilli and streptococci inoculated before administering antitoxin were in some cases definitely, but not markedly, larger than those produced by either organism alone.

(2) The progress of the diphtheritic infections, whether alone or in combination with the streptococci, was *promptly arrested by antitoxin*.

(3) The addition of charcoal suspension to either the streptococci or the diphtheria bacilli did not result in an enhanced reaction.

#### USE OF TOXIN INSTEAD OF *C. DIPHTHERIAE*

Since the infections were controlled by antitoxin, which in itself does not inhibit the growth of diphtheria bacilli, it seemed probable that the most important factor in the enhanced reactions mentioned in conclusion 1, above, was the exotoxin which presumably was formed and fixed in the tissues *before* the antitoxin was administered. Therefore, it seemed possible that the antitoxin, by its neutralization of toxin produced *after* its administration, had arrested all the reactions, since these would depend for their further evolution on continued elaboration of free toxin by organisms growing in the tissues. This would spoil the experiments by killing the animals quickly. To eliminate this possibility a technique was developed which did not require the use of diphtheria organisms or antitoxin, e.g., cultures of *C. diphtheriae* were replaced by sterile toxin. Since the identity of toxins from all strains of *C. diphtheriae* appears to have been demonstrated by Parish *et al.* (1932), Povitsky *et al.* (1933), Zinnemann and Zinnemann (1939), and Zinnemann (1946), the substitution of PW no. 8 toxin for that produced *in vivo* by other strains of *C. diphtheriae* (C1, C2, etc.) seems to need no special justification. It was decided to use a constant amount of toxin despite the fact that the experimental conditions were thereby made less like a normal synergistic association of two living organisms.

The use of a standard toxin dosage was advantageous in that it permitted careful control of this factor. It was disadvantageous in that it provided a fixed amount of toxin in the tissue at one time, whereas the living diphtheria cultures supplied continuous small amounts, although when the diphtheria cultures were used there was an unknown and variable amount of toxin taking part in the reaction.

The toxin (T106) was diluted so that 0.1 ml, the dose used, contained just enough toxin to cause a slight necrosis on intracutaneous inoculation in normal rabbits.

The materials for injection were as follows:

(1) *Toxin control*. Toxin dilution plus a volume of the nutrient base (plain or blood infusion broth) equal to that in which the particular dose or organism under study was suspended.

(2) *Organism control*. Culture plus an equal volume of Moloney and Taylor diluent (1932). The toxin was diluted in Moloney and Taylor diluent.

(3) *Combined*. Toxin dilution plus an equal volume of culture.<sup>5</sup> All inocula were equal in volume.

<sup>5</sup> The plain or blood infusion broth in the toxin control and the Moloney and Taylor diluent in the organism controls equalized the volumes of these substances in the combination. The undiluted toxin was in an infusion broth base and, therefore, the diluted toxin preparation contained infusion broth diluted with Moloney and Taylor diluent to approximately 1 in 200. This small amount of nutrient was disregarded in the preparation of the culture controls.

More than 30 organisms were studied in combination with diphtheria toxin using this technique.

#### RESULTS

With most of the test organisms the reactions produced in combination with diphtheria toxin were regularly identical with, or only slightly different from, those produced by the organism or toxin alone.<sup>6</sup> With some of the test organisms, the areas of erythema, edema, and necrosis produced by the combination with diphtheria toxin were sometimes larger than those produced by either alone.<sup>7</sup>

The tests with *Diplococcus pneumoniae* type I were especially interesting. In three animals inoculated intradermally in the manner described above, enormous areas of erythema and very marked and extensive edema developed at the site of inoculation of the combination of the diphtheria toxin and pneumococcus culture, but not at either control site. One of the three animals died in 3 days. However, similar extensive reactions (with occasional deaths) developed in some rabbits at the pneumococcus control site and not at the toxin pneumococcus site. In three animals inoculated with heated<sup>8</sup> toxin and pneumococcus cultures similar results were obtained: extensive reactions developed in one animal at the pneumococcus control site only; in another at the toxin pneumococcus site only; and in the third at both the pneumococcus control and toxin pneumococcus sites. Finally, to dissociate the toxin from the phenomenon entirely 13 rabbits were inoculated with a pneumococcus culture alone. Three of these 13 animals developed the typical extensive reaction, and 1 died in 7 days. Only small abscesses developed in the other rabbits. These observations are in accord with those of Goodner (1928) and of Abernethy (1937) on the effect of type I pneumococci on rabbits.

In view of the fact that in some cases an extensive reaction originated from one pneumococcus inoculation and not from another in the same animal, it seems probable that the difference between the various responses was due solely to variations in local tissue resistance, minor alterations in injection technique, or both.

#### DISCUSSION

In this section of the investigation 32 different species of bacteria were injected into rabbits in combination with diphtheria toxin. The association of 19 of these species with the toxin did not result in any significant difference between the reactions produced by the tested combinations and the reactions produced by the organisms or toxin alone. With 13 of the organisms, the

<sup>6</sup> *Hemolytic streptococci* (beta type), Lancefield groups A and B; *streptococcus* (alpha type); *streptococcus* (gamma type); *H. influenzae* (type unknown); avirulent *C. diphtheriae*, gravis and mitis types; *C. pseudodiphthericum*; *D. pneumoniae*, types I and II; *N. catarrhalis*; *N. intracellularis*; *N. gonorrhoeae*; an unidentified, gram-positive, biscuit-shaped diplococcus from a throat culture; and a species of *Lactobacillus* from the trochea of a fatal can of malignant diphtheria.

<sup>7</sup> *Streptococcus* (beta type), groups B and G; *S. aureus*; *H. influenzae*, type B; *C. xerose*; *Klebsiella* sp.; *E. coli*; *E. typhosa*; *N. sicca*; and *D. pneumoniae*, type III.

<sup>8</sup> At 70 C for 10 minutes.

reactions at the sites of inoculation of the combined preparations were larger in one or more of the rabbits than those produced by the toxin alone or the organism alone. These occurred so irregularly that their significance with respect to synergism seems doubtful.

There was no correlation between the type, species, or genus of the organisms and the property of developing enhanced reactions in association with diphtheria toxin. Organisms from several entirely unrelated genera exhibited this property, but not all species of the same genus, or even types of the same species, did so. For example, enhanced reactions occurred with 1 or more species of *Streptococcus*, *Hemophilus*, *Escherichia*, etc., but with only 1 of 4 species of *Neisseria* and with only 2 of 3 strains of hemolytic streptococci (beta type), group B.

The studies with one strain of pneumococcus type I indicated that variations in host resistance or in local tissue resistance, slight alterations in injecting technique, or all three, were responsible for marked differences in the reactions produced by those organisms. It seems likely that most of the differences in the tests with other bacteria were due to the same factors. The possibility is not denied, however, that the association of the toxin with some of these organisms may have been in some way responsible for the development of enhanced reactions.

Since this study was initiated to investigate bacterial synergism with regard especially to malignant diphtheria, it is interesting to consider the possible significance of the reactions described above in relation to that disease. The ratio of severity of reaction produced by the toxin or organisms alone to those produced by combinations was in no wise comparable to the ratio of severity of ordinary diphtheria to that of malignant diphtheria. Furthermore, in the experiments in which antitoxin was administered before the test injections were given, the enhanced reactions did not develop, indicating complete control of the diphtheritic intoxication, whereas malignant diphtheria is characteristically antitoxin-refractory. In view of these facts and the variety and number of organisms which exhibited the enhanced reaction with diphtheria toxin, it seems unlikely that synergism has any great significance in malignant diphtheria.

It is recognized, however, that conditions prevailing in the human disease are different from, and probably more complex than, those in these experiments. In the first place, the differences in the host and the site of infection preclude any direct comparison, and, in the second place, most of the experiments were carried out with a fixed amount of toxin, whereas in the human disease there are living diphtheria bacilli producing a continuous supply of small amounts of toxin. Therefore, though the possibility of a significant degree of synergistic action between diphtheria bacilli (or toxin) and other organisms in human infections is not eliminated, the evidence obtained from the experiments herein described in no way supports it.

#### SUMMARY AND CONCLUSIONS

The problem of the etiology of malignant diphtheria was investigated with reference to the possibility of a synergistic action between *Corynebacterium*

*diphtheriae* and hemolytic streptococci or other organisms. Two experimental procedures were employed: (1) intraperitoneal inoculation, into mice, of combinations of living cultures of homologous pairs (both organisms isolated from the same case of malignant diphtheria) of *C. diphtheriae* and streptococci (beta type); and (2) intracutaneous inoculation, into rabbits, of diphtheria bacilli or toxin in combination with living cultures of various organisms.

In the experiments with mice deaths occurred in greater numbers and in a shorter time among those animals receiving combinations of sublethal doses of diphtheria bacilli and streptococci than among those receiving the same dose of either organism alone. This was at first interpreted as indicative of a synergistic action between the two organisms. However, similar results were obtained with three of the same strains of streptococci when sterile infusion broth, nonspecific particulate matter, or both were substituted for the cultures of diphtheria bacilli. It was concluded that the volume or quality of nutrient material or inert particles, or both, in the inoculum has a marked effect on the lethality of streptococci for mice. In three experiments in which these factors were adequately controlled no synergism was apparent between two homologous pairs of *C. diphtheriae* and hemolytic streptococci (beta type), group B. It is probable that the factors of pabulum and particulate matter were responsible for all the results and that no synergism occurred between any of the strains of *C. diphtheriae* and streptococci used.

The experiments with rabbits in which nonspecific factors in the inocula were controlled revealed no reactions that could definitely be attributed to synergism between diphtheria toxin and one or more strains of 32 different bacteria.

No clear-cut evidence has been obtained of a true synergism between *C. diphtheriae*, or its toxin, and a variety of other organisms.

Interpretation of the results of experiments on bacterial synergism must be made with due regard to the influence of nonspecific factors, which were found to modify significantly the apparent virulence of some of the organisms used in this investigation.

The problem of the etiology of malignant diphtheria is as yet unsolved. Further investigations are essential to determine whether the factors of etiological significance are related to the diphtheria bacillus or its products, to host factors, or to bacterial synergism.

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# THE EFFECT OF SODIUM ACENAPHTHENE(5)SULFONATE ON A STRAIN OF *EBERTHELLA* TYPHOSA

THOMAS H. GRAINGER, JR., AND DOROTHY L. WILMER

*Department of Biology, Lehigh University, Bethlehem, Pennsylvania*

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In recent years acenaphthene has been found to cause polyploidy in plants. Shmuck (1938), Kostoff (1938*a*, 1938*b*), Navaskin (1938), and Shmuck and Gus-seva (1939) have indicated that acenaphthene may be superior to colchicine for inducing mutations in higher plants. Ark (1946) reported that induced, per-manent mutations were formed in broth saturated with acenaphthene in *Phyto-monas michiganensis* and *Erwinia carotovora*. Luria (1947) reports, however, that Ark's results suggest selection for mutants rather than induction of muta-tions. The present report is concerned with the effect of an isomeric sulfonate of acenaphthene on a strain of *Eberthella typhosa*. This aromatic compound is sodium acenaphthene(5)sulfonate.

## METHODS AND RESULTS

The strain of *Eberthella typhosa* used in this study had been isolated from the blood of a typhoid patient. A single cell of this culture was isolated and put into nutrient broth for a previous study concerning the effects of X-rays on this strain (Grainger, 1947). This single cell isolation of this culture was used for this study as well. The strain was characteristic of the species in respect to all the bio-chemical and physiological characteristics as described in Bergey's *Manual* (1939). Neither its antigenic formula nor its phage specificity was determined.

The culture was subcultured daily into nutrient broth as well as plated on nutrient agar by the streak method for 14 successive days before being used in the study. Observations were made on the colonial character after 24-hour incubation on nutrient agar. The colonies were studied by means of a colony microscope lens (3 ×) to note any change in morphology. The colony character of the strain remained a constant smooth type.

A 2 per cent aqueous solution of sodium acenaphthene(5)sulfonate<sup>1</sup> was made and sterilized. The final reaction was pH 6.8. Various amounts of this solution were added to 10 ml of nutrient broth (pH 7.2) to determine the amount needed to inhibit the growth of this strain of *Eberthella typhosa*. One loopful (4 mm) of a 24-hour nutrient broth culture was added to each tube containing the differ-ent concentrations of the compound in nutrient broth. It was found that as high as 5 ml of the 2 per cent solution in 10 ml of nutrient broth failed to inhibit the growth of the strain of *Eberthella typhosa*.

It was of interest to note whether sodium acenaphthene(5)sulfonate would have any effect on the colonial character of this culture. Thus, to a flask containing

<sup>1</sup> The sodium acenaphthene(5)sulfonate was kindly supplied by Prof. R. T. Wendland, Chemistry Department, Lehigh University.

95 ml of sterile nutrient broth were added 5 ml of a sterile, aqueous, 2 per cent solution of this compound. This amount of the compound in the nutrient broth was 0.1 per cent. The final pH was 7.0. One loopful of the 24-hour nutrient broth culture of *Eberthella typhosa* was added to this flask. A loopful was likewise added to a flask containing 100 ml of nutrient broth. This served as a control. The flasks were placed in the incubator at 37 C.

Subcultures on nutrient agar plates by the streak method were made daily for 30 days from the flask containing the compound in the nutrient broth as well as from the control nutrient broth. The colonies were studied by means of a colony microscope (3 X) to note any changes in morphology. At least 100 well-isolated colonies were studied daily on the nutrient agar obtained from the subcultures from each flask.

The colonies from the flask of nutrient broth containing the sodium acenaphthene(5)sulfonate showed no changes until the seventh day. At this time, 12 colonies out of 100 examined were of the R type. From this time on the daily subcultures showed a percentage of rough forms that varied between 2 and 18. The percentage of the R type varied from day to day, but this was probably due to the chance in isolating the rough forms which had appeared on the seventh day. The R type did not displace the original S type. The period of observation ended at 30 days.

The R colony of *Eberthella typhosa* was picked and placed in nutrient broth for further study. It was found to be characteristic of the species in respect to all the biochemical and physiological characteristics as described in Bergey's *Manual* (1939), except for one difference: it would not ferment the monosaccharide, galactose. The R colony character of the strain remained constant after repeated subcultures over a period of 3 months.

There was little variation in the colonies from the control nutrient broth. Occasionally an intermediate colony was observed. All the other colonies showed a typical smooth type of colony. This observation has been noted with this culture for a period of over 6 months.

Since it is desirable to avoid any implication concerning the hereditary mechanism of bacteria until we have more adequate knowledge, the term *anomalous variation* has been suggested by Grainger (1947). The modification of this culture by the use of this compound is an anomalous variation, as well as any other variation the causative mechanism of which is unexplained at the present time. It is possible that the anomalous variation that occurred in this study may have been spontaneous and that the environment favored the growth of the resistant organisms to this compound. It has been shown repeatedly, for example, that bactericidal and bacteriostatic substances act as selecting agents to permit the detection of resistant strains. As Luria (1947) has warned, "One should be particularly cautious before claiming induction of mutation by environmental agents when the change appears to affect the whole population exposed. It is very likely that in such cases a type arisen by spontaneous mutation has completely displaced the original type because of favorable selection by the special environment."



On the other hand, the compound may have played a role in producing the anomalous variation. The substance did not appear to be bacteriostatic in the concentration used and the R type that appeared on the seventh day did not displace the original S type. The percentage of rough colonies did not seem to increase from the time they first made their appearance until the period of observation ended 23 days later. Thus, it would seem that the anomalous variation that occurred was due either to the effects of this compound or merely to a spontaneous change.

#### ACKNOWLEDGMENT

The authors wish to express their appreciation to Professor Stanley Thomas for the interest and advice he has given throughout this work.

#### SUMMARY

One-tenth of a per cent of sodium acenaphthene(5)sulfonate in nutrient broth produced an anomalous variation from a smooth culture of *Eberthella typhosa*. A rough type colony was first observed after the seventh day of incubation. The percentage of rough colonies varied daily between 2 and 18, from this period until the observations ended at 30 days. The rough type did not displace the original S culture.

The rough strain of *Eberthella typhosa* was found to be characteristic of the parent S strain in respect to all the biochemical and physiological characteristics, except that it did not ferment galactose.

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# THE INITIAL BODY AND THE PLAQUE FORM IN THE CHLAMYDOZOACEAE

GEOFFREY RAKE

*Division of Microbiology, The Squibb Institute for Medical Research,  
New Brunswick, New Jersey*

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Examination with the light microscope and any of several differential stains has failed to resolve the exact significance of the so-called initial bodies and plaque forms which, together with the elementary bodies, represent stages in the developmental cycles of the *Chlamydozoaceae* (lymphogranuloma-psittacosis group of agents; Rake, 1947). Previous studies from this laboratory (Rake and Jones, 1942) suggested that the initial bodies, particularly of the agent of lymphogranuloma venereum, are in fact larger single forms of the agent and that they divide as such in the early stages of the developmental cycle, but it was not clear whether the whole unit is larger or whether one is dealing with a single elementary body enveloped in a wide mantle of "capsular" substance. It seemed clear from differential staining with Noble's stain that the later plaque forms represent, rather than single forms, colonies of elementary bodies embedded in a "capsular" matrix. The elementary bodies themselves stain red, while the "capsular" material is green.

## THE INITIAL BODY

Recently doubt has been thrown on the very existence of these initial bodies (Kurotckin *et al.*, 1947), and it has been suggested that they represent merely reaction products, perhaps of a lipid nature, developed as a result of the reaction of the yolk cells to the invasion of the infectious agent.

In the past year we have had occasion to take many electron micrographs of different species of *Chlamydozoaceae*, mostly of the agent of feline pneumonitis. The techniques used and part of the results of these studies have been published elsewhere (Rake *et al.*, 1946; Hamre *et al.*, 1947). As these reports have indicated, micrometric measurements of the diameters of the bodies seen and photographed with the electron microscope show, for the unshadowed bodies, a range of from 350 to 580  $m\mu$  with a mean of 455  $m\mu$ , and for the gold-shadowed bodies a range of from 303 to 728  $m\mu$  with a mean of 525  $m\mu$ . Approximately normal distribution curves were obtained in both series. However, it was noted (Rake *et al.*, 1946) that occasional larger forms were seen, and an unshadowed body with a diameter of 770  $m\mu$  was recorded.

Since then this problem has been studied further. The 2,374 elementary bodies of the agent of feline pneumonitis available for measurement in all the micrographs taken of this agent have been scrutinized. A total of 5 unshadowed bodies (out of 501) and 8 gold-shadowed bodies (out of 1,873) gave measurements sufficiently far outside the previously recorded range as to seem significant.

Thus in the unshadowed group three measured 710 m $\mu$ , one 740 m $\mu$ , and one 770 m $\mu$ . In the shadowed group one measured 780 m $\mu$ , one 800 m $\mu$ , two 830 m $\mu$ , one 840 m $\mu$ , one 860 m $\mu$ , and two 920 m $\mu$ . Representative examples are shown in figure 1, nos. 1 to 4.

It is believed that these bodies lie so far on the high side of the range of size distribution of diameter, shown to be normal, as to be significant. They certainly would appear larger in the light microscope than the commonly accepted elementary bodies and would thus fill the criterion for initial bodies. It is true they are few in number—only 0.5 per cent in the present series—but this proportion is certainly of the same order of magnitude as the proportion of initial bodies making up the viral suspensions prior to drying them on the collodion membranes.

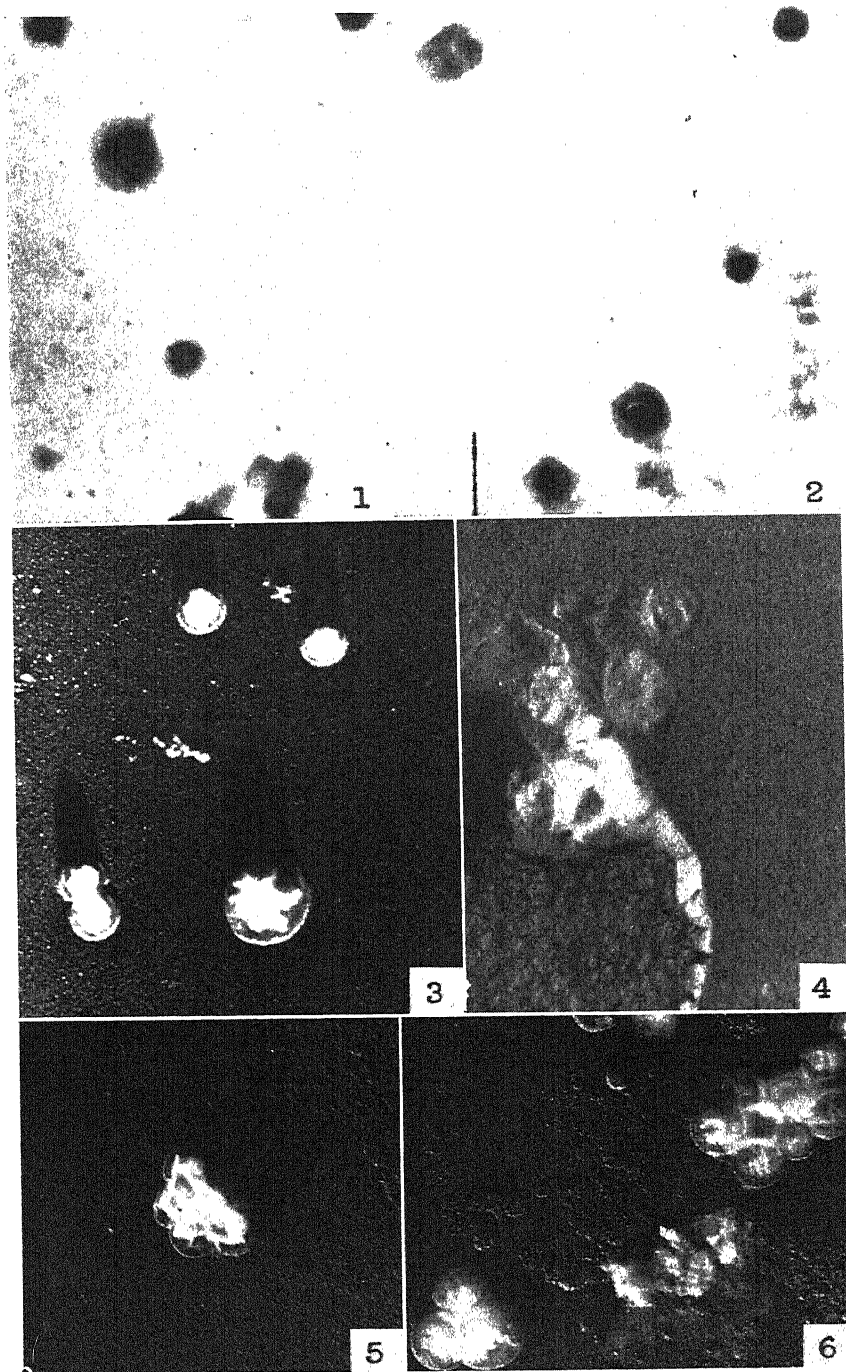
#### THE PLAQUE

As has been pointed out above, previous studies (Rake and Jones, 1942) had indicated that the so-called plaques represented colonies of elementary bodies embedded in a "capsular" matrix. Examination of the series of electron micrographs of the agent of feline pneumonitis showed groups of bodies which could be interpreted as colonies, since the close juxtaposition of the bodies and the molding together of their contiguous surfaces would favor such an hypothesis rather than one of secondary agglutination (figure 1, nos. 5 and 6). It is true that no surrounding "capsular" matrix was to be observed, but this is not surprising. As has been shown elsewhere (Rake and Jones, 1942), smear preparations even from yolk cells shown by section to be loaded with plaques never show any such plaques in an intact state, even with the light microscope, and fragmenting plaques are rare. If this is the case with the more gentle techniques involved in preparation for examination under the light microscope, the failure to demonstrate any intact "capsular" matrix in the present preparations is not surprising.

#### SUMMARY

Examination of electron micrographs of the agent of feline pneumonitis has demonstrated the existence of large bodies lying well outside the range of size found for the elementary bodies. Such large bodies form approximately 0.5 per cent of all bodies studied. They are believed to represent initial bodies. It is also possible to demonstrate closely packed groups of elementary bodies which are presumed to represent the colonies of elementary bodies usually found in sections of infected yolk sac embedded in a "capsular" matrix to form the

- 
- No. 1. Elementary bodies and one initial body not shadowed with gold. 14,220  $\times$ .
  - No. 2. Elementary bodies and two initial bodies not shadowed with gold. 14,650  $\times$ .
  - No. 3. Elementary bodies and one initial body; gold-shadowed, 21.7 mg of gold, angle 11°32', 10-cm distance. 14,455  $\times$ .
  - No. 4. Replicas of one elementary and one initial body; gold-shadowed, 1.5 mg of gold, angle 18°26', 9-cm distance. 14,140  $\times$ .
  - No. 5. Small group of elementary bodies; gold-shadowed, 21.7 mg of gold, angle 11°32', 10-cm distance. 14,220  $\times$ .
  - No. 6. Two groups of virus particles; gold-shadowed, 25.2 mg of gold, angle 10°59', 10.5-cm distance. 14,500  $\times$ .



plaque. It is believed that the matrix itself is easily disintegrated and so disappears during preparation of the screens for the electron microscope, a thesis in accord with other observations.

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# THE RELATIVE ERRORS OF BACTERIOLOGICAL PLATE COUNTING METHODS

THOMAS L. SNYDER

*Camp Detrick, Frederick, Maryland*

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It is generally conceded that the degree of precision ordinarily attained with dilution plate counting methods of enumerating viable bacteria is not so great as could be desired. The extent of variation encountered even under the best conditions is considerably larger than the maximum permitted, for example, of routine chemical analyses. Coefficients of variation consistently as high as 30 per cent of the mean have been reported (Mudge and Lawler, 1928; Ziegler and Halvorson, 1935), although considerably lower values have also been encountered (Jennison, 1937). Some of the principal sources of variation which may be identified at the present time are:

(1) The presence of clumps, consisting of various numbers of organisms, in the original suspension (Jennison, 1937).

(2) The error involved in preparing a dilute suspension (Jennison and Wadsworth, 1940).

(3) The error involved in measuring aliquots of this suspension into plates.

(4) The distribution of organisms in these aliquots (Fisher, Thornton, and Mackenzie, 1922; James and Sutherland, 1939; Sutherland and James, 1938; Wilson and Kullmann, 1931).

(5) Factors which influence the development of the inoculated cells into visible colonies (Harmsen and Verweel, 1936-1937; McNew, 1938).

The relative importance of these different sources of error depends considerably upon the conditions of experimentation; but it appears probable that if species of bacteria characterized by persistent or tenacious clumping are excluded, the contribution of the first two sources listed above may be reduced through careful technique to a small part of the total. It is not an uncommon practice to describe the variability of replicate plates as the total error of the plate count, and this is probably not often a serious misrepresentation. However, methods of estimating "dilution error" have been described (Jennison and Wadsworth, 1940) and are undoubtedly valuable for precise studies.

The sum of the variations due to the three remaining sources may be estimated from the observed variability of replicate plates. The chief purpose of the present study is an analysis of this sum, separating the variation related to the measurement of aliquots into plates from the sum of the remaining two factors, and estimating its relative importance. The data required for this purpose were obtained by comparing several measuring methods with respect to the volumes of suspension delivered and the plate counts obtained with each method. This problem does not appear to have been investigated specifically, although methods for increasing the precision of measurement have been devised and recommended

for use (Miles, Misra, and Irwin, 1938; Wilson, 1922). Since the comparison was also designed to assist in the selection of appropriate bacterial counting methods for practical use, several modifications, which are not strictly essential to the principal objective, were included and assessed.

#### METHODS

Three methods of measuring aliquots of inoculum were employed, and these, together with other modifications of technique, provided five plating methods for comparison, as follows:

A. Prepared and dried plates were inoculated with 6 drops of a known dilution of organisms delivered by means of a single calibrated capillary pipette.<sup>1</sup> The drops were allowed to fall at well-separated points on the surface of the agar.

(1) The area covered by the individual drops was increased by repeatedly tilting the plate in all directions. This method is commonly used with *Brucella* species.

(2) The inoculum was distributed over the entire surface of the plate with a sterile, bent glass rod.

B. Prepared and dried plates were inoculated with 0.1-ml quantities of dilute suspension delivered by means of a single "exax" serological 1-ml pipette graduated in tenths and hundredths of a ml. The inoculum was distributed by means of a sterile, bent glass rod. This method has been used routinely with *Bacterium tularense* (Snyder, Engley, Penfield, and Creasy, 1946) and is a modification of the method of Anderson and Stuart (1935).

C. Nine-tenths ml of dilute suspension were delivered by means of a single "exax" 1-ml serological pipette into the following:

(1) Sterile, empty plates, which then received 15 ml melted agar medium held at 45 C. The medium and inoculum were mixed and allowed to solidify. This is the conventional poured plate, with the single exception that an attempt was made to increase accuracy by avoiding delivery of the final 0.1 ml of the inoculum.

(2) Culture tubes (16 by 150 mm) containing 2 ml of melted 3/2 strength agar medium held at 45 C. The medium and inoculum were mixed and allowed to solidify on the walls of the tube by rotating the tube in a horizontal position under the cold water tap. This is the rolled tube method (see Wilson, 1922) modified with respect to the size of the inoculum.

In order to provide a valid basis for comparison, a single bacterial species was counted with all five methods. *Shigella dysenteriae*, strain 14-4, was selected for this purpose because its rather simple nutritional requirements and its relative indifference to oxidation-reduction potential could be expected to permit abundant growth in all cases.

Final dilutions for plating were prepared from 24-hour stock cultures in tubes of nutrient broth, using as the diluent 0.2 per cent Difco gelatin in 1.0 per cent disodium phosphate (12H<sub>2</sub>O) adjusted to pH 6.8. Whenever the same dilution

<sup>1</sup> We are indebted to M/Sgt. D. E. Drukenmiller, Jr., for a supply of these pipettes, which were prepared essentially according to the directions of Donald (1915).



could be used for several plating methods, all samples were taken from a single bottle of that dilution.

Tryptone agar of the following composition was used in all plates:

Difco tryptone .....	2.0 per cent
Sodium chloride .....	0.5 per cent
Glucose .....	0.5 per cent
Difco agar .....	2.0 per cent
Adjusted to pH 7.0 to 7.2	

In the case of method C2 (rolled tubes), all ingredients were originally present in concentrations 1.5 times those indicated, but the addition of the inoculum reduced them to approximately those listed.

All surface-inoculated plates were dried before being used by storage at 34 C for 3 to 5 days. This is not recommended as a method for producing uniformly dried plates, but it is believed that the extent of drying is a relatively unimportant factor in the case of *S. dysenteriae*.

Plates and tubes were incubated at 34 C for 24 hours after inoculation. Plates were counted with the aid of a Quebec colony counter, whereas rolled tubes were counted over oblique illumination, each colony being marked with a wax pencil. Counting rolled tubes was found to be extremely tedious.

The volumes of fluid delivered by means of the three pipetting methods were determined gravimetrically. Approximately 60 replicates were delivered into weighed sample bottles by each method, the exact technique used in plating being followed as nearly as possible. The bottles were closed immediately after the addition and weighed to the nearest one-tenth of a milligram. However, in order to reduce the total number of weighings required, distilled water was substituted in these measurements for gelatin-phosphate diluent, so that the bottles might be merely dried, rather than cleaned and reweighed, before being used again. This method appeared to be valid, since the difference in weight of the dry sample bottles before and after completion of all measurements averaged only +0.7 mg, and in only one case (apparently an error in the initial weighing) did it exceed 2.0 mg, or 2 per cent, of the smallest volume measured.

The substitution of distilled water necessitated a correction for the volume of gelatin-phosphate diluent delivered by the capillary-dropping pipette method (A), since the volumes of the drops formed are influenced by the surface tension of the fluid delivered. The correction factor required was determined by counting the number of drops of distilled water and diluent delivered by the particular capillary pipette used throughout. The pipette was mounted vertically in a fixed position, and the drop rate was controlled by means of a capillary air inlet. A constant volume was assured by horizontal markings on the pipette, and the portion of the last drop delivered was estimated to the nearest one-tenth of a drop. The results were quite constant with water, averaging for 11 determinations  $102.05 \pm 0.05$  drops. They were equally constant for any one sample of gelatin-phosphate diluent, but different samples differed appreciably: the average of 5 determinations on each of 6 samples was  $105.56 \pm 0.18$  drops. The differ-

ence between water and diluent is highly significant. On the basis of these averages the ratio of volume of drops of diluent to volume of an equivalent number of drops of water is 0.96675. This is considerably less than the theoretical ratio calculated from surface tension measurements and probably may be accounted for by the small diameter of the pipette tip.

#### STATISTICAL METHODS

In order to compare the magnitude of plate counts obtained by different methods of plating, the observed mean counts were adjusted according to the dilution and volume of suspension inoculated. In all cases the adjusted mean count is that expected with 0.1 ml of the  $10^{-5}$  dilution.

Standard deviations,

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{N - 1}}$$

( $x$  = counts of individual plates,  $\bar{x}$  = mean count, and  $N$  = number of plates), were derived from the unbiased estimate of the second central moment and are thus independent of the particular distribution function with which the counts may conform.

In order to determine the significance of differences between adjusted mean counts, standard errors,

$$s_{\bar{x}} = \frac{s}{\sqrt{N}},$$

were adjusted to conform with the adjustment of the means:

$$\text{adjusted } s_{\bar{x}} = \frac{s_{\bar{x}}(\text{adjusted } \bar{x})}{\bar{x}}.$$

This adjustment is dependent upon the assumption that a strict proportionality exists between the standard error and the mean, and may be only approximately correct. Consequently, most confidence may be placed in tests of significance of differences between means when the required adjustment is minimal.

The relative error of the different plating methods was compared on the basis of the coefficient of variation,

$$V = \frac{s}{\bar{x}}.$$

This definition of the coefficient of variation is that of Cramér (1946), and it differs from the usual percentage representation by a factor of 1/100. The standard deviation of the coefficient of variation,

$$s_V = \sqrt{\frac{s^2}{2N\bar{x}^2} \left( 1 + 2 \frac{s^2}{\bar{x}^2} \right)},$$

is also due to Cramér (1946).

Tests of significance of differences between the adjusted mean counts of differ-

ent plating methods, and between coefficients of variation of different methods, were based on Student's ratio,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_{\bar{x}_1})^2 + (s_{\bar{x}_2})^2}}$$

or

$$t = \frac{V_1 - V_2}{\sqrt{(s_{V_1})^2 + (s_{V_2})^2}}$$

Values of  $t$  exceeding the 5 per cent level of the  $t$ -distribution (1.960) for an infinite number of degrees of freedom are considered significant, those exceeding the 1 per cent level (2.576) highly significant.

TABLE 1

*Analysis of gravimetric measurements of water volumes delivered by three pipetting methods*

STATISTICS	METHODS OF PIPETTING*		
	A	B	C
Number of replicates.....	60	60	59
Mean weights of water (grams).....	0.1801	0.1025	0.9087
Calculated volumes of diluent (ml)†.....	0.1741	0.1025	0.9087
Standard deviations.....	0.0036	0.0028	0.0090
Standard errors.....	0.0005	0.0004	0.0012
Coefficients of variation.....	0.02001	0.02727	0.00989
Standard deviations of coefficients of variation.....	0.00183	0.00249	0.00090

\* Methods of pipetting:

A. Six drops delivered with capillary pipette.

B. One-tenth ml delivered with 1-ml serological pipette.

C. Nine-tenths ml delivered with 1-ml serological pipette.

† Calculations based on the ratio of drop volumes of distilled water and gelatin-phosphate diluent.

#### EXPERIMENTAL RESULTS

The results of gravimetric measurements of volumes delivered by the three pipetting methods are shown in table 1. The weights may be converted directly into volumes without appreciable error, since the density of water at 25 C is 0.9970 grams per ml (Hodgman and Holmes, 1940). It may be noted that the capillary pipette used delivers in 6 drops 0.180 ml of distilled water or 0.174 ml of gelatin-phosphate diluent. The serological pipettes delivered slightly in excess of the volumes intended, and presumably the volumes were the same in the case of both water and diluent.

The variability of even the most inaccurate method (B) was small, the coefficient of variation being less than 3 per cent of the mean.<sup>1</sup> The coefficients of variation necessarily included errors of weighing, but these were apparently small. The variabilities of the three pipetting methods, however, differed appreciably. The order of increasing error was C, A, B in the ratio of 1:2:2.7. It

may be shown that the difference between any two of these coefficients of variation was statistically significant and that method C was more accurate to a highly significant degree than either of the other two. It may be concluded, therefore, that of the methods tested most precise delivery was obtained when 0.9 ml were delivered from a 1-ml pipette, and the least precise when only 0.1 ml was delivered from the same type of pipette. When it is necessary to use small inocula of the order of 0.1 to 0.2 ml, the capillary pipette provides more accurate delivery than do 1-ml pipettes.

In order to compare the variabilities of the three pipetting methods with the total error of the plate count, of which they are a part, two plating experiments were conducted using the five plating methods described. The two experiments were essentially repetitive. In experiment 1 plates were inoculated by methods

TABLE 2  
*Analysis of plate counts (experiment 1)*

STATISTICS	PLATING METHODS*			
	A1	A2	B	C1
Number of replicates.....	66	64	67	49
Mean counts (colonies per plate).....	293.14	308.86	178.34	153.37
Adjusted mean counts†.....	168.36	177.39	173.91	168.77
Standard deviations.....	21.949	22.928	13.999	11.727
Standard errors.....	2.7017	2.8660	1.7104	1.6753
Adjusted standard errors‡.....	1.5517	1.6460	1.6679	1.8435
Coefficients of variation.....	0.07488	0.07423	0.07850	0.07646
Standard deviations of coefficients of variation.....	0.00655	0.00660	0.00683	0.00777

\* For plating methods, see text.

† Values expected with 0.1 ml of  $10^{-5}$  dilution.

‡ Adjusted to conform with adjusted mean counts.

A1, A2, and B from the  $10^{-5}$  dilution, and plates and tubes by methods C1 and C2, respectively, from the  $10^{-6}$  dilution, of a single culture. In this experiment the rolled tube counts were low and extremely erratic, presumably because of having been held in the water bath at 45 C for 10 to 20 minutes after inoculation, and therefore they were not included in the analysis. In experiment 2, which employed a second culture, the dilutions used with each method were so chosen that approximately equal mean counts could be expected with all methods. These dilutions were  $0.6 \times 10^{-5}$  in the case of methods A1 and A2,  $1.0 \times 10^{-5}$  with method B, and  $0.11 \times 10^{-5}$  with methods C1 and C2. Furthermore, in this experiment rolled tubes were rolled and cooled immediately after inoculation, a procedure requiring the attention of two operators.

The analyses of these two experiments are shown in tables 2 and 3. Since rather similar values were obtained for the adjusted mean counts of the various methods, as well as for their variabilities as indicated by coefficients of variation (which may legitimately be compared), it is necessary to resort to statistical tests

to determine the significance of the differences between them. The significances of the differences between all possible combinations of adjusted mean counts

TABLE 3  
*Analysis of plate counts (experiment 2)*

STATISTICS	PLATING METHODS*				
	A1	A2	B	C1	C2
Number of replicates.....	56	56	56	56	56
Mean counts (colonies per plate).....	152.91	161.36	158.30	159.05	167.48
Adjusted mean counts†.....	146.37	154.46	154.37	159.11	167.54
Standard deviations.....	12.807	13.568	11.513	14.157	12.338
Standard errors.....	1.7115	1.8132	1.5386	1.8919	1.6488
Adjusted standard errors‡.....	1.6382	1.7356	1.5003	1.8926	1.6494
Coefficients of variation.....	0.08376	0.08409	0.07273	0.08901	0.07367
Standard deviations of coefficients of variation.....	0.00797	0.00800	0.00691	0.00848	0.00700

\* For plating methods, see text.

† Values expected with 0.1 ml of  $10^{-5}$  dilution.

‡ Adjusted to conform with adjusted mean counts.

TABLE 4  
*Significance of differences between mean counts of various plating methods*

EXPERIMENT	METHODS* COMPARED	DIFFERENCES BETWEEN ADJUSTED MEANS	STANDARD ERRORS OF DIFFERENCES	STUDENT'S† RATIO
1	A1, A2	-9.03	2.262	3.992(++)
	A1, B	-5.56	2.278	2.440(+)
	A1, C1	-0.41	2.410	0.171
	A2, B	+3.47	2.343	1.482
	A2, C1	+8.62	2.471	3.487(++)
	B, C1	+5.14	2.486	2.070(+)
2	A1, A2	-8.09	2.387	3.389(++)
	A1, B	-8.00	2.221	3.602(++)
	A1, C1	-12.74	2.503	5.091(++)
	A1, C2	-21.18	2.325	9.110(++)
	A2, B	+0.09	2.294	0.038
	A2, C1	-4.66	2.568	1.813
	A2, C2	-13.09	2.394	5.467(++)
	B, C1	-4.74	2.415	1.964(+)
	B, C2	-13.18	2.230	5.910(++)
	C1, C2	-8.43	2.510	3.359(++)

\* For plating methods, see text.

† Ratios exceeding the 5% level of the *t*-distribution (1.960) are considered significant (+), those exceeding the 1% level (2.576), highly significant (++).

within each of the two experiments are shown in table 4. Since these experiments were not designed primarily for the comparison of mean counts, probably only a

few of the tabulated comparisons are valid. The significance of the difference between means of plates inoculated from different dilutions, or even from different dilution bottles, should be questioned because the error involved in the preparation of dilutions was not determined. Methods A1, A2, and B in experiment 1 are not subject to this criticism. It may be seen that the adjusted mean counts of methods A2 and B were not significantly different, whereas both were significantly greater than that of method A1. The higher counts are undoubtedly related to the more efficient spreading of the inoculum which was obtained in methods A2 and B by the use of a bent glass rod. It is suggested that the opportunity for the formation of congruous colonies is decreased by this procedure. The only comparisons of mean counts within experiment 2 which may not be questioned are those between methods A1 and A2 and between methods C1 and C2, each pair of which was inoculated from a single dilution bottle. Again, method A2 gave a significantly higher count than method A1, confirming the results of the first experiment. In addition, the adjusted mean of method C2 (rolled tubes) was significantly higher than that of method C1. This confirms a similar observation of Wilson (1922), and the magnitude of our difference (5 per cent) is approximately the same as his.

On the other hand, it would appear legitimate to compare the variabilities of any two plating methods within either experiment, or even those of different experiments, since the coefficient of variation is generally considered independent of the original culture, of the dilution used, and of the mean count. Actually, however, independence between the coefficient of variation and the mean count follows from the assumption that the standard deviation is strictly proportional to the mean. Since this may be only approximately correct, most confidence may be placed in comparisons between the variabilities of the plating methods of the second experiment because the unadjusted means are nearly equal in this case. This consideration is relatively unimportant, however, because it may be shown that no two of the coefficients of variation obtained in both experiments differed significantly. The 36 possible combinations may be covered by a single test, using the greatest difference observed (that between methods B and C1, experiment 2) and the smallest observed standard deviation of the coefficient of variation (method A1, experiment 1). Student's ratio then becomes

$$t = \frac{0.01628}{\sqrt{2(0.00655)^2}} = 1.757,$$

which is nonsignificant and at least as great as that of any pair of observed values. It may be concluded, therefore, that the variability or error of any one of the five plating methods tested was not significantly greater than that of any of the others.

It is now necessary to reconcile this conclusion with the finding that the errors of the three pipetting methods used did differ significantly. This may be done most conveniently by considering the components of error involved. The coefficients of variation of the plate counts will be regarded for this purpose as the total errors ( $T$ ), consisting in part of variabilities due to the pipetting of aliquots

into plates, which are represented by the coefficients of variation ( $P$ ) of the appropriate pipetting methods as determined gravimetrically. The remaining variabilities ( $R$ ) which complete the total are probably attributable to the distribution of organisms in the aliquots and to factors influencing the development of the organisms inoculated. Since the addition theorem is defined for terms of the same order as the variance ( $s^2$ ), the relation between these components may be expressed by the equation,

$$T^2 = P^2 + R^2,$$

and  $R$  may be determined from the equation,

$$R = \sqrt{T^2 - P^2}.$$

TABLE 5

*Analysis of error involved in various plating methods*

EXPERIMENT	PLATING METHOD*	COEFFICIENTS OF VARIATION			$T - R$	$\frac{100 (T - R)}{T}$
		Total† ( $T$ )	Pipetting‡ ( $P$ )	Remainder§ ( $R$ )		
1	A1	0.07488	0.02001	0.07216	0.00272	3.63
	A2	0.07423	0.02001	0.07148	0.00275	3.70
	B	0.07850	0.02727	0.07361	0.00489	6.23
	C1	0.07646	0.00989	0.07515	0.00131	1.71
2	A1	0.08376	0.02001	0.08134	0.00242	2.89
	A2	0.08409	0.02001	0.08168	0.00241	2.87
	B	0.07273	0.02727	0.06742	0.00531	7.30
	C1	0.08901	0.00989	0.08846	0.00055	0.62
	C2	0.07367	0.00989	0.07300	0.00067	0.91

\* For plating methods, see text.

† Coefficients of variation taken from tables 2 and 3.

‡ Coefficients of variation taken from appropriate entries in table 1.

§  $R = \sqrt{T^2 - P^2}$ . Additional preliminary analysis of this remainder variation ( $R$ ),<sup>a</sup> subtracting the variance due to a hypothetical Poisson distribution of organisms in aliquots of suspension, shows that residual variation is small and, in some cases, negative. There is at this time no plausible explanation for these negative residuals, but they suggest that there is something in the method which tends to keep the counts at an artificially even level, which would somewhat impair their use as measures of variation of bacterial populations.

The values of these statistics are given in table 5 for all the plating methods of experiments 1 and 2. It will be seen that the remainder ( $R$ ) constitutes almost the total error ( $T$ ). The final column of this table lists the percentage contributions of the pipetting errors to the total errors when reduced to terms of the same order as the actual measurements. Taking both experiments into account, this amounted to only 2.9 to 3.7 per cent in the case of capillary pipetting methods, to only 6.2 to 7.3 per cent in the case of 0.1-ml aliquots delivered with a 1-ml pipette, and to only 0.6 to 1.7 per cent in the case of 0.9-ml aliquots. This would appear to explain sufficiently the failure of the more precise methods of measurement to influence significantly the total error of the plate count.

## DISCUSSION

Experimental results have been presented which indicate that, within the limits of precision ordinarily employed in pipetting aliquots of inoculum into individual plates, the accuracy of measurement provides a negligible contribution to the total error, and that even so crude a method as that of measuring 0.1-ml quantities with a 1-ml pipette does not significantly increase the plating error. Since a conclusion of this sort naturally leads to the recommendation of changes in the technique of plate counting, it would be well to subject the experimental evidence to careful scrutiny.

In the first place, the statistical analysis appears to be valid throughout. Essentially identical experiments, each employing numbers of replicates generally considered sufficient to determine the pertinent statistics, were mutually confirmatory. Estimates of error were independent of the form of distribution function which may be involved, and in at least one experiment the possible influence of unequal means was minimal. Tests of significance were necessarily based on a normal distribution function, but it is known that even with markedly skewed distributions the statistics concerned are approximately normally distributed.

Also, it might be objected that the relative familiarity of the operators with the different techniques introduced a bias in favor of a particular method. It is especially probable that the coefficient of variation reported for the capillary-dropping pipette method is greater than that which might be obtained by an experienced operator. However, this issue cannot be considered critical for the present study. Since the most inaccurate pipetting method did not appreciably affect the total plating error, further improvement of the relatively precise capillary-dropping method would, at most, have no greater effect.

The specific coefficients of variation reported apply only under the experimental conditions described and with the particular organism used. The principal conclusion which is drawn from them, however, can be affected only by conditions or a bacterial species which leads to a *reduction* of the total error. The extent to which the total error must be reduced in order that the pipetting error may exert an appreciable effect depends upon the magnitude of the pipetting error. The limiting value ( $T_p$ ) of the total error corresponding to various percentage contributions ( $p$ ) of the pipetting error ( $P$ ) is found by solution of the equations,

$$p = \frac{100(T - R)}{T}$$

and

$$R = \sqrt{T^2 - P^2},$$

which is

$$T_p = \frac{P}{\sqrt{\frac{p}{100} \left( 2 - \frac{p}{100} \right)}}.$$



Values of  $T_p$  corresponding to various  $p$  are shown in table 6 for the three pipetting methods used. It may be seen that an appreciable contribution (for example, 20 per cent of the total error) from the pipetting error is not attained until the total error is reduced to 0.016 to 0.045, depending on the pipetting method used. It is questionable whether or not such low values for the total error are ever attained, in any real sense, with bacteriological plate counts. Although coefficients of variation as low as 0.01 have been reported<sup>2</sup> for individual counts derived from small numbers of plates, such occurrences are related to the characteristic fluctuation of the statistic with small samples and cannot be regarded as accurate estimates. Determinations based on large samples, or averages of numerous small samples, are rarely lower than our reported values.

TABLE 6

*Limiting values of total error ( $T_p$ )\* corresponding to various percentage contributions of pipetting error*

PIPETTING ERROR PERCENTAGE OF TOTAL ( $p$ )†	METHODS OF PIPETTING‡		
	A	B	C
1	0.1419	0.1934	0.0701
5	0.0641	0.0874	0.0317
10	0.0459	0.0625	0.0227
20	0.0334	0.0454	0.0165
33	0.0270	0.0368	0.0133
50	0.0231	0.0315	0.0114

$$* T_p = \frac{P}{\sqrt{\frac{p}{100} \left( 2 - \frac{p}{100} \right)}}$$

$$† p = \frac{100(T - R)}{T}$$

‡ Methods of pipetting:

A. Six drops delivered with capillary pipette ( $P = 0.02001$ ).

B. One-tenth ml delivered with a 1-ml serological pipette ( $P = 0.02727$ ).

C. Nine-tenths ml delivered with a 1-ml serological pipette ( $P = 0.00989$ ).

Since, then, there is every reason to believe that the conclusion derived from the experimental results is valid for general application, it is clear that efforts to improve the accuracy of bacteriological plate counting methods should at present be directed elsewhere than toward increased precision of measuring aliquots into plates. Two plausible sources of the remaining variability were indicated in the introduction. One of these, the distribution of organisms in the aliquots, has received considerable attention (Fisher, Thornton, and Mackenzie, 1922; James and Sutherland, 1939; Sutherland and James, 1938; Wilson and Kullmann, 1931) and is presumably susceptible to precise mathematical definition. If this is the case, the contribution of this particular source of variability may be reduced

<sup>2</sup> This figure is calculated from a value given by Jennison (1937) for the standard deviation of the mean (standard error) of 5 plates, expressed as percentage of the mean.

to whatever extent desired by sufficiently increasing the number of plates inoculated. It may be assumed that the standard error of replicate plates is approximately inversely proportional to the square root of the number of plates from which it is determined. Therefore, whatever the error obtained with the conventional usage of 3 plates, for example, it may be halved by the use of 12 plates or reduced by  $\frac{2}{3}$  or  $\frac{3}{4}$  by the use of 27 or 48 plates, respectively. Investigators are discouraged from making full use of this relationship by the inconvenience and expense involved in the preparation, inoculation, and counting of large numbers of plates. The present study provides some encouragement in this direction, in that it justifies the use of a plating method (B) with which large numbers of plates (9 to 18) may be as rapidly and conveniently inoculated as could small numbers (3 to 5) with standard methods. In addition, preparation of the plates is facilitated because they may be prepared on a large scale at convenient intervals and stored.

The other source of variability indicated in the introduction is more difficult to assess, but it has been shown (Harmsen and Verweel, 1936-1937) that in certain cases, at least, conditions that might be expected to favor the development of the inoculated bacteria also tend to bring the plate counts into better agreement with the Poisson distribution function; hence this type of study might be used to assess the suitability of various media. It would appear that this possibility merits more extensive investigation.

In view of all these considerations it is possible to make recommendations with respect to that part of the dilution plate count procedure subsequent to the preparation of dilutions. When the growth requirements of the bacterial species permit, prepared and dried plates should be inoculated with 0.1-ml aliquots delivered by means of 1-ml serological pipettes, and the inoculum should be distributed over the surface with a sterile glass rod. This method is quite as precise as any of those studied and has the advantage that one source of variability may conveniently be decreased by approximately 42 or 59 per cent by increasing the number of plates inoculated from 3 to 9 or 18. Using method B of the present study for an example, a standard error of 4.37 per cent of the mean count is provided by 3 plates, is reduced to 2.52 per cent with 9 plates, and to 1.78 per cent with 18 plates. These figures, like all the data presented, are exclusive of any error contributed by the process of preparing dilutions. In the case of an organism apparently characterized by a larger variability, as for example *Bacterium tularensis* (Snyder, Engley, Penfield, and Creasy, 1946), the reduction of the standard error would be more impressive.

It is recognized that frequently practical limitations with respect to the number of plates inoculated are set by considerations of economy instead of manipulative convenience, so that the foregoing recommendations may well be referred principally to studies in which precision is of especial importance, rather than to routine practice. Even in the latter case, however, use of the surface plating technique with the usual number of 3 to 5 plates may be recommended on the basis of saving time and effort.

The rolled tube method may be recommended in cases where strict economy of

medium and of space for incubation is important. There is suggestive evidence that this method will give a higher, and therefore a presumably more accurate, count. In our opinion, however, this method is not sufficiently convenient for large-scale studies.

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#### SUMMARY

Five bacteriological plate counting methods were compared with respect to the magnitude and reliability of the counts obtained with *Shigella dysenteriae*.

The coefficients of variation of the five plating methods were not significantly different, even though the coefficients of variation of the three methods of measuring aliquots of inoculum into plates did differ significantly. This phenomenon was referred to the relative magnitudes of the plating errors (coefficients of variation = 0.0727 to 0.0890) and of the pipetting errors (coefficients of variation = 0.0099 to 0.0273). It was demonstrated that no significant contribution to the total error can be expected from the pipetting methods used unless the total error is considerably less than that observed in this study.

Efficient spreading of the inoculum in the case of surface plating methods significantly increased the counts obtained. The rolled tube method gave a significantly higher count than the poured plate method.

As a result of these findings it is possible to recommend more extensive use of surface plate counting methods, which are conducive to greater replication of plates.

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## CULTURAL STUDIES ON THE YEASTLIKE PHASE OF *HISTOPLASMA CAPSULATUM* DARLING

S. B. SALVIN

*Division of Infectious Diseases, National Institute of Health, Bethesda, Maryland*

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DeMonbreun (1934) and Hansmann and Schenken (1934) were the first to report the growth in artificial culture of the etiological agent of histoplasmosis, *Histoplasma capsulatum* Darling. DeMonbreun was notably successful in culturing both growth phases of the fungus: the mycelial phase, on glucose agar at room temperature; and the yeastlike phase, on 10 per cent rabbit blood agar in sealed tubes at 37 C. This yeastlike phase, which appears in the parasitic condition in the human or animal host, is obtained in culture by inoculation of the culture medium directly from infected animals, since, with the exceptions of the strains studied by Ciferri and Redaelli (1934) and by Conant (1941), conversion of the mycelial to the yeastlike phase has not been demonstrated as occurring outside the animal body. Once the yeastlike phase is obtained, however, it may be maintained in most strains by repeated transfer every 5 to 14 days on 10 to 20 per cent blood agar at 37 C.

As described by DeMonbreun (1934), Conant (1941), and others, the colony of the yeastlike form on blood agar slants is white, moist, and of pasty consistency. The individual organism is a spherical or oval cell, 1.5 to 3 by 2 to 3.5 microns in diameter, with a thin, enveloping capsule. Reproduction occurs by the formation of a single bud at the more pointed end of the cell.

A few studies have been conducted on the cultural characteristics of the mycelial form, but very little has been done on the yeastlike phase. Moore (1935) compared the mycelial growth of two strains on about 15 different types of agar or broth. Howell (1940, 1941) studied the growth rates of the mycelium under varying temperatures and hydrogen ion concentrations. DeMonbreun, on the other hand, conducted some cultural studies on the yeastlike phase. Having originally isolated this latter phase from a mycelium and yeast cell mixture at the bottom of flasks of infusion broth (pH 7.2), he was successful in growing the oval cells on several types of solid media at 37 C, namely, on whole blood agar, fresh serum agar, Loeffler's blood serum, and Petragnani's medium (without malachite green). The maintenance of a proper degree of moisture and of a temperature of 37 C was considered of utmost importance. The nature of the culture medium also played an important role in determining the character of the growth, for when the yeastlike phase was inoculated onto glucose agar, Sabouraud's agar, potato, gelatin, or bread, notwithstanding the degree of moisture or temperature, the resulting growth was mycelial. Although attempts were made to culture the yeastlike phase in liquid media at 37 C, such as in infusion broth, serum, or infusion broth with 1 per cent serum, consistent growth without the development of mycelia was not obtained.

The primary stimulus to the following experiments was a need for a method whereby large numbers of the yeastlike cells of *H. capsulatum* could be easily obtained. A fluid medium in which the yeastlike phase would multiply rapidly and extensively and not revert to the mycelial form would present distinct advantages over blood agar slants. Accordingly, investigations were initiated to determine more fully the cultural requirements and characteristics of the yeastlike phase, and thereby to develop a liquid medium for its maximum growth.

#### MATERIALS AND METHODS

The 19 strains of *H. capsulatum* used were all obtained from the collection maintained in this laboratory. Saline suspensions of 3-to-4-month-old Sabouraud's agar cultures of mycelium and conidia were injected intraperitoneally into mice. After 2 to 5 weeks the mice were killed and the yeastlike phase of all but two of the strains was recovered on 20 per cent rabbit blood agar slants from the liver, spleen, or kidney. This phase was maintained at 37 C on 20 per cent rabbit blood agar slants in tubes which had been sealed to prevent drying of the agar. The cultures were transferred once a week, although every 2 to 3 weeks proved satisfactory for the maintenance of the yeastlike phase. In the following investigations, the fungus was grown at 37 C in 10 ml of the semisolid medium (to be described) in a 25-by-150-mm tube, which in most cases was sealed with paraffin to prevent evaporation.

#### EXPERIMENTS AND RESULTS

Since the yeastlike phase was known to exist, and possibly grow, in the blood of man and animals, and since blood and blood serum had been shown by De-Monbreun to maintain the yeastlike phase, a strain of *H. capsulatum* (6521) was inoculated into several liquid media in which sheep serum or plasma was the basic substance. Although cultures in each of these media were incubated at 37 C for 3 weeks under different oxygen tensions, no marked growth of the yeastlike phase was apparent.

Many other variations of the medium were tried, but in all there appeared very little, if any, growth of the yeastlike phase. However, when the viscosity of the medium was increased by the addition of 0.175 per cent Difco agar, the yeastlike phase grew most abundantly and was nearly free of mycelium. The term "nearly free" is used since examination of several slides containing the yeastlike phase would reveal only an occasional abortive hypha. The medium on which this profuse growth appeared had the following composition (Difco products): proteose peptone, 10 g; neopeptone, 3.25 g; tryptone, 3.25 g; glucose, 2.0 g; sodium chloride, 5.0 g; disodium phosphate, 2.5 g; agar, 1.75 g; and distilled water to make 1,000 ml. This medium was the basic one used in most of the ensuing experiments and will be referred to hereafter as "YP medium."

*Hydrogen ion concentration.* The YP medium was dispensed in 10 ml amounts in 25-by-150-mm culture tubes, and duplicate tubes were adjusted to pH readings of 3.9, 4.3, 4.85, 5.4, 5.85, 6.3, 6.9, 7.05, 7.3, 7.5, 7.7, 8.1, 8.6, and 9.6. The tubes

were then inoculated with the yeastlike phase of strain 6521 and incubated at 37 C for 3 weeks.

The results showed maximum growth of the yeastlike phase at pH's between 6.3 and 8.1. In these tubes, as well as those in which less growth occurred, almost no mycelium was found. The experiment was repeated with two other strains, and similar results were obtained.

*Temperature.* In order to determine the optimum temperature for growth of the yeastlike phase, 7 strains of *H. capsulatum* were grown at 4 temperatures, 25, 31, 37, and 43 C, in YP medium. The inoculum in all cases was the yeastlike phase.

At 25 C and at 31 C the resultant growth was mycelial in character, whereas at 37 C the yeastlike phase grew extensively with no mycelium evident. At 43 C, with the exception of one strain which showed extensive growth, the yeastlike phase grew much less abundantly than at 37 C, although again no hyphae appeared. Accordingly, of the temperatures studied the best growth of the yeastlike phase occurred at 37 C.



FIG. 1. GROWTH OF THE YEASTLIKE PHASE OF STRAIN 6521 OF *H. CAPSULATUM* AT pH 7.3 IN YP MEDIUM IN AGAR PERCENTAGES OF 0.0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, AND 0.5, (FROM LEFT TO RIGHT)

*Viscosity.* In tubes containing 10 ml of the basic YP medium, the percentage of agar was varied from 0.0 to 0.5 as follows: 0.0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 per cent. Each of these percentages was inoculated in duplicate with the yeastlike phase of strain 6521 and incubated at 37 C.

After 3 weeks' incubation examination of the cultures revealed marked development of the yeastlike phase in the tubes containing an agar percentage of 0.1 and higher. Virtually no growth was discernible in the tubes with agar percentages of 0.05 and less. The most cells developed in the medium containing 0.2 per cent agar, with slightly less in the one having 0.1 per cent agar. The yeastlike colonies in the YP media with agar percentages of 0.3 to 0.5 were extensive and also unmixed with mycelial elements, but, owing probably to the solidity of the medium, they were restricted to the very surface of the medium (figure 1). Additional studies at agar concentrations between 0.1 and 0.3 per cent indicated that 0.175 per cent agar provided a viscosity for the most extensive development of the yeastlike phase, to the exclusion of the mycelial phase.

These experiments to determine the optimum viscosity of the medium for the growth of the yeastlike phase of *H. capsulatum* were repeated with 5 other strains, and results similar to those obtained with strain 6521 were obtained. When

growth was extensive, it appeared in the upper portion of the medium (figure 1). When growth was markedly restricted, as in the tubes with no agar, no yeast cells were apparent in the upper part of the medium, although very small quantities developed at the bottoms of the tubes.

The reason for the luxuriant growth of the yeastlike phase in a medium with a low percentage of agar was obscure. Two possible explanations were tested: (1) the agar contained an essential growth substance, or (2) the agar produced a narrow zone of a particular oxygen or carbon dioxide tension essential for the development of the yeastlike phase. Experiments were conducted to test these two hypotheses.

Difco agar, which was the type used in the foregoing experiments, was washed for 48 hours in slowly running tap water and then incorporated into tubes of YP medium in a percentage of 0.175. These tubes and similar ones without agar were inoculated in duplicate with 12 strains of *H. capsulatum* and incubated at 37 C for 3 weeks. Again, in all cases, growth of the yeastlike phase was luxuriant in the tubes of 0.175 per cent agar and virtually nonexistent in those containing no agar.

In order to test the possibility that some inorganic element was exerting an effect on the growth of the yeastlike phase, as much as ten times the concentration of agar used in the YP medium, namely up to 1.75 g per 100 ml of medium, was ashed and added to the medium. The tubes were then inoculated with 7 strains and incubated at 37 C for 3 weeks. In no case did the yeastlike phase appear.

To eliminate completely the possibility of there being some necessary organic or inorganic growth substance in the agar, semisolid "silica gel" was prepared according to the method of Anderson and MacSween (1942), with the exception that a ratio of one part silicate solution to 25 parts of nutrient medium was employed instead of the 1:9 ratio recommended. The purpose of the latter modification was to produce a medium of approximately the same viscosity as one containing 0.175 per cent agar. The nutrient base was YP medium in all cases. The silica gel semisolid medium, after inoculation with 14 strains and incubation at 37 C for 3 weeks, brought about luxuriant growth of the yeastlike phase in all cases but one, with virtually no mycelial fragments discernible.

*Oxygen requirements.* Since the yeastlike phase grew near the surface of the YP medium, the possibility presented itself that the organism concerned was strongly aerobic and that the semisolid YP medium served to keep the cells on or near the surface of the medium. Accordingly, experiments were initiated to determine the oxygen requirements of the cells.

Tubes containing 10 ml of the YP medium were inoculated with the yeastlike phase and incubated at 37 C under each of the following conditions: (1) in complete anaerobiosis (produced by a suction pressure of 30 inches of mercury), (2) under one-half inch of sterile liquid petrolatum, (3) under 10 per cent carbon dioxide in air, (4) under 20 per cent carbon dioxide in air, (5) under 40 per cent carbon dioxide in air, (6) under 80 per cent carbon dioxide in air, and (7) under 100 per cent oxygen.



The 5 strains subjected to conditions of complete anaerobiosis grew about as extensively as the controls exposed to ordinary room air at 37 C. Under sterile liquid petrolatum the same 5 strains multiplied again only in the yeastlike phase, although to a slightly lesser degree than the controls. Under 10, 20, 40, and 80 per cent carbon dioxide, luxurious development of the yeastlike phase was observed in all cases, with virtually no abortive hyphae present. When in an atmosphere of 100 per cent oxygen, growth of the yeastlike phase was somewhat less than the controls, with one strain having a noticeable amount of mycelial development.

In addition, a flask with YP medium minus agar was inoculated with the yeastlike phase and shaken constantly and vigorously for 4 weeks at 37 C. No extensive growth of the yeastlike phase was then apparent. However, if the inoculum of yeastlike cells was carefully floated on cork shavings, oil drops, or paraffin chips on the surface of YP medium minus agar, marked growth of the yeastlike phase resulted on the surface of the medium.

*Age of culture.* The yeastlike phase of *H. capsulatum* was maintained in culture both on 20 per cent rabbit blood agar slants and in YP medium. The cultures on the blood agar were transferred every 7 days, whereas those in the YP medium were transferred only once a month. Nevertheless, several times isolates on the blood agar slants spontaneously reverted to the mycelial phase and had to be restored by inoculations from the YP medium. This spontaneous transformation to mycelium at no time occurred in the YP medium. Thus, it would seem that the semisolid medium is more suited for the maintenance of the yeastlike phase in culture.

*Growth of other species on YP medium.* Since the yeastlike phase of *H. capsulatum* grew so well on YP medium, it seemed logical to inoculate other species of pathogenic fungi therein to obtain their respective parasitic phases. The species studied in this regard were *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Sporotrichum schenckii*.

When the yeastlike phase of *B. dermatitidis* from rabbit blood agar slants was inoculated into the YP medium and incubated at 37 C, the 5 strains tested developed primarily as mycelium. In the studies on 6 strains of *C. immitis*, with inocula consisting of either a saline suspension of conidia or a suspension of spherules from an infected mouse testis, there was no obvious development of the yeastlike phase in the YP medium at 37 C. Of the 6 strains of *S. schenckii* incubated in the YP medium, two developed entirely in the yeastlike form, but the others appeared both in the mycelial and budding phases.

#### SUMMARY

Seventeen strains of *Histoplasma capsulatum* were grown in a fluid medium in the yeast-like phase.

The best growth occurred at hydrogen ion concentrations between 6.3 and 8.1, at a temperature near 37 C, and in a medium containing a mixture of organic nitrogen compounds.

No growth appeared in the "YP medium" unless a small percentage of agar, silica gel, oil, or some similarly functioning substance was added.

The oxygen and carbon dioxide tensions were of little importance.

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## NOTES

### A NOTE ON FORMATE RICINOLEATE LACTOSE BROTH

PIERRE FREDERICQ AND MAX LEVINE

*Iowa State College, Ames, Iowa*

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In the formate ricinoleate medium recommended in *Standard Methods of Water Analysis*, gas production may be due to the decomposition of either lactose or the salt of formic acid in the medium. In case the latter is the source of gas, the reaction of the medium turns alkaline, whereas if the former (lactose) is decomposed an acid reaction will result. In the standard methods formula no indicator to determine the reaction of the medium is included, with the result that considerable additional work is necessary in order to ascertain whether the gas former is probably a lactose fermenter.

In the course of collateral studies, it was noted that all cultures of *Escherichia*, *Citrobacter*, and *Aerobacter* produced acid and gas, whereas those of the genera *Proteus*, *Salmonella*, and the paracolon bacilli produced gas with an alkaline reaction when formate ricinoleate broth was employed.

In *Standard Methods for Water Analysis*, 9th edition, it is pointed out, in conjunction with the completed test, that if gas is formed in formate ricinoleate broth, inoculated from an agar slant showing sporeformers and gram-negative rods, the probable presence of the coliform group of organisms should be verified by inoculation from the formate ricinoleate to a tube of standard lactose broth and to a new agar slant. The objective here is to determine whether the gas produced was due to lactose fermentation. If, however, an indicator were present in the formate ricinoleate broth, this last step could be dispensed with as far as detection of a lactose-fermenting organism is concerned.

*Standard Methods*, furthermore, states that, if in this last step spores are present on the agar slant, then for all practical purposes organisms of the coliform group may be considered absent. In view of the fact that gram-negative rods were originally present on the agar slant from which the formate ricinoleate broth tube was inoculated and that the paracolon bacilli, *Proteus*, and *Salmonella* produce gas with an alkaline reaction in formate ricinoleate broth, the conclusion that typical coliforms are absent when spores are found on the agar slant, though correct, may (if gram-negative rods are still present with the spores) actually result in missing the presence of *Salmonella* and the slow lactose-fermenting paracolon bacilli, which might actually be of sanitary significance.

It is suggested, therefore, that the simple addition of an acid-base indicator in the formate ricinoleate medium might serve to eliminate further work when both acid and gas are produced, and the objective is merely to detect typical coliform bacteria, whereas the production of an alkaline reaction in conjunction with gas would serve to facilitate detection of *Salmonella* and paracolon bacilli, if present.

# EOSIN METHYL-GREEN SULFITE AGAR: A MODIFICATION OF LEVINE'S E.M.B. AGAR

PIERRE FREDERICQ<sup>1</sup>

*Iowa State College, Ames, Iowa*

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Most of the differential media for enteric bacteria are based on the fermentation of lactose. The splitting of this carbohydrate gives rise to acid as well as oxidizing products which change the color of the indicators employed. As the lactose-containing media give a definite advantage for the growth of the lactose-fermenting coli-aerogenes group, many of the media are supplemented with selective inhibitors (appropriate concentrations of bile salts, brilliant green, etc.). As a result of this, one has to employ different media for best results in the isolation of either *Escherichia*, *Salmonella*, or *Shigella*. There is, therefore, a need for a medium on which *Escherichia*, *Salmonella*, and *Shigella* would grow well, but on which *Escherichia* would not profit too much by its lactose-fermenting ability.

Levine's E.M.B. agar is probably the best medium for the isolation and characterization of the coli-aerogenes group, but the high toxicity of the methylene blue prevents the growth of some members of the genera *Salmonella* and *Shigella*. Taking advantage of the fact that aniline dyes are much less toxic in their reduced (colorless) form (Dubos: J. Exptl. Med., **49**, 575), methyl green reduced by sodium sulfite was employed to replace the methylene blue of Levine's E.M.B. The low toxicity of the reduced methyl green permits a good growth of the lactose-negative enteric strains. Furthermore, the advantage experienced by the lactose-fermenting strains on this lactose-containing medium is counterbalanced by the oxidation of the methyl green and its subsequent increase in toxicity against the lactose fermenters.

The eosin methyl-green sulfite (E.M.G.S.) agar is prepared in the following manner:

- |  |          |
|--|----------|
| A. To distilled water.....   | 1,000 ml |
| Add: proteose peptone.....   | 10 g     |
| lactose (Difco).....   | 25 g     |
| 1% solution of methyl green.....   | 15 ml    |
| B. Decolorize (until only faint color remains) with a 10% solution of sodium sulfite, adding a drop at a time (will require 1.5 to 2.0 ml per liter of media). |          |
| C. Add: 2% solution of eosin Y.....  | 7.5 ml   |
| agar.....  | 15 g     |
| D. Boil to dissolve completely and sterilize in the autoclave at 15 pounds' pressure (121 C) for 15 minutes.   |          |

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<sup>1</sup> Fellow of the Belgian American Educational Foundation.

(Note: It is important to add the constituents in the order listed as it has been observed that some samples of agar interfere with the proper reduction of the methyl green.)

This medium, at the same time, indicates pH and rH changes: without sodium sulfite, it is equivalent to Levine's E.M.B., and without eosin it is equivalent to Endo's medium. It gives, therefore, a wide range of different shades among lactose-fermenting as well as lactose-nonfermenting colonies. Changes in color, resulting from the fermentation of lactose, do not diffuse around the colony as in Endo's medium but are localized to the center of the fermenting colony as in Levine's E.M.B. The appearance of the coli-aerogenes strains on this medium is similar to that on E.M.B., and lactose-negative strains form larger colonies with shades varying from gray to red according to the alkalinity produced. *Shigella dysenteriae* strains, which failed to grow on Levine's E.M.B., gave good-sized colonies on this medium. Except for enterococci, which produce very small colonies, E.M.G.S. agar completely inhibits gram-positive organisms.

# ANAEROBIC OXIDATION OF HYDROCARBONS BY SULFATE-REDUCING BACTERIA<sup>1</sup>

WILLIAM D. ROSENFELD<sup>2</sup>

*Scripps Institution of Oceanography, University of California, La Jolla, California*

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Assimilation of aliphatic hydrocarbons by anaerobic bacteria has been noted in the case of *Desulfovibrio* species by Novelli and ZoBell (J. Bact., **47**, 447). The following data reveal that these sulfate reducers catalyze the anaerobic oxidation of a considerable variety of mixed hydrocarbons. Substrates were emulsified in water with gum arabic, autoclaved, mixed with sterile mineral salts solution, and inoculated with mixed cultures of sulfate-reducing anaerobes. Cultures were incubated in 60-ml glass-stoppered bottles at 27 C. Hydrocarbon oxidation was accompanied by sulfate reduction, which was employed as an index of bacterial activity. Sulfates were not reduced in the presence of gum arabic mixtures containing no hydrocarbons.

SUBSTRATE	NUMBER OF CULTURES TESTED	NUMBER THAT WERE ACTIVE
Crude oils:		
Calif. crude no. 138-4.....	27	27
Calif. crude no. 143-3.....	27	27
Calif. crude no. 144-1.....	27	26
Pa. crude no. 148-2.....	29	28
Pa. crude no. 148-4.....	29	29
Refinery products:		
Kerosine no. 109-5.....	29	28
Lubricating oil no. 140-2.....	27	26
Paraffin oil.....	27	26
Gum arabic (control).....	29	0

Long-chain aliphatic hydrocarbons have undergone rapid destruction by sulfate reducers. Insoluble fatty acids were isolated as intermediates in this consumption. The presence of fatty acids, however, was transitory, for they underwent further degradation. The utilization of hexadecane was vigorous and was traced quantitatively by the ether extraction procedure of Gould (Science, **98**, 546) and the fractionation methods of Wilson and Hansen (J. Biol. Chem., **112**, 457). A typical experiment, employing culture XXIX:130-1, is recorded below.

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<sup>2</sup> Present address: California Research Corporation, La Habra, California.

DAYS OF INCUBATION	EXTRACT FRACTIONS	
	Unsaponifiable (mg/L)	Fatty acid (mg/L)
0	910	0
13	568	120
30	48	0

Hydrocarbon utilization by *Desulfovibrio* species appears to be associated with the presence of a dehydrogenase system. This enzymatic activity has been detected by employing washed cells of pure sulfate-reducing species in the Thunberg method. Substrates such as hexadecane or hexadecene are well suited to the procedure and, upon activation, readily donate hydrogen to methylene blue. Nonsulfate-reducing anaerobes known to be incapable of the cultural utilization of hydrocarbons were included as controls. Tubes prepared without hydrocarbon demonstrated no dehydrogenation of the gum arabic normally used to emulsify the substrates.

SUBSTRATE	SULFATE REDUCERS		NONSULFATE REDUCERS	
	Tested	Active	Tested	Active
Hexadecane.....	3	2	4	0
Hexadecene-1.....	3	3	4	0
Gum arabic (control).....	3	0	4	0





# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## SOUTHERN CALIFORNIA BRANCH

LOS ANGELES, CALIFORNIA, MAY 22, 1947

IN VIVO ANTIBACTERIAL ACTIVITY OF HEXENOLACTONE. Charles W. Johnson and J. W. Bartholomew, Department of Bacteriology, University of Southern California.

The antibacterial agent hexenolactone occurs naturally in whole oranges, baked whole meal bread, and ungerminated cereal. This compound was recently synthesized by Meddwar, Robinson, and Robinson (1943) and Kuhn and Jerchel (1943). Bartholomew and Hervey (1947) demonstrated the *in vitro* inhibitory effect of this compound at varying concentrations against a variety of pathogenic and nonpathogenic gram-positive and gram-negative bacteria. Particular effectiveness was noted in the case of *Salmonella enteritidis*.

The first experiment in this *in vivo* study was designed to determine the LD<sub>50</sub> of hexenolactone for 14- to 18-gram white mice. The chemical was diluted in physiological saline and administered by the intraperitoneal route. Death was used as an end point of toxicity. All observations were made at the end of a 96-hour period. The LD<sub>50</sub> of hexenolactone calculated according to the method of Reed and Muench (1937) was 5.26 mg per 14- to 18-gram mouse. A 1-mg dose of hexenolactone was selected as the therapeutic dose.

Experiments using varying dosages of *Salmonella enteritidis* and a single 1-mg injection of hexenolactone administered 30 minutes after injection of organisms by the intraperitoneal route showed protection ranging from 0 per cent, at the highest dosage of organisms, to 100 per cent, at the lowest dosage of organisms. Using a constant dosage of approximately 100,000 organisms, protection ranged from 30 to 60 per cent for the treated mice.

An unsuccessful attempt was made to increase the rate of survival, using *Salmonella enteritidis*, by administering 1 mg of hexenolactone 30 minutes after injection

of the organisms and 1 mg 3 hours after the initial injection of the chemical. Preliminary observations indicate a possible decrease in the rate of survival with increased amounts of the chemical.

REPORT OF A RELATIVELY SEVERE AND PROTRACTED DIARRHEA PRESUMEDLY DUE TO *SALMONELLA PULLORUM* FROM THE INGESTION OF INCOMPLETELY COOKED EGGS. T. F. Judefind, Department of Pathology and Bacteriology, College of Medical Evangelists, Loma Linda, California.

Although *Salmonella pullorum* had formerly been considered nonpathogenic for man, a number of cases of human diarrhea from which this organism has been isolated have been reported. Attention has recently been drawn to this subject by the report of Mitchell *et al.* (J. Infectious Diseases, 79, 57-62, 1946), which gives data on a major food-poisoning outbreak characterized by a diarrhea averaging 2 to 3 days and involving 423 persons, 172 of whom required hospitalization. *Salmonella pullorum* was rather definitely incriminated in this outbreak, and the available evidence indicated that the source of the organism was incompletely cooked eggs in rice pudding.

A case is reported of a female, age 29, who developed a diarrhea 8 days after hospital entry for obstetrical care. This diarrhea lasted for about a month. Just before recovery there was an acute exacerbation associated with a temperature of 102.6 F that required a second hospitalization. *Salmonella pullorum* was isolated from the patient's stool at the onset of the diarrhea. The source of the infection appeared to be incompletely cooked eggs, which were served for breakfast each morning while the patient was hospitalized for obstetrical care.

ELECTRONIC PRESERVATION OF BOSTON BROWN BREAD. *J. W. Bartholomew and R. G. Harris*, Department of Bacteriology, University of Southern California.

One hundred 1-pound loaves of packaged Boston brown bread were each inoculated with 100,000 spores of a mixture of *Penicillium* and *Aspergillus* molds. Each loaf was then placed in a dielectric field produced by equipment engineered by the Electronic Chemical Engineering Company of Los Angeles. This equipment was designed to operate at 5,000 volts, 200 milliamperes, and at a frequency of approximately 26 megacycles. Each loaf was heated to a temperature of 150 F before removal from the field. This took about 3 minutes. None of the inoculated and heated loaves were moldy after 3 weeks of observation. All of the control loaves were moldy by the third day. Considerable trouble with "arching" and resultant burning of the bread was experienced. This could probably be eliminated by correct electrode design. The slowness of the process could be eliminated by the use of more powerful equipment.

Although Boston brown bread is not a conductor, it was decided to see what the effect would be if the bread were put in an induction coil. This resulted in much more rapid heating, and the "arching" and burning were easily prevented. Heating was

only on the surface of the bread, but molding seldom is a problem in the interior. Of 100 loaves inoculated with mold and heated in the induction coil, none were moldy after 3 weeks of observation, whereas all of the controls were moldy within 3 days. This method was superior to dielectric heating.

Bread treated by the foregoing methods retained all its original moisture and flavor. The wrapper was not affected.

CHEMOTHERAPY OF TUBERCULOSIS IN INTRAVENOUSLY INFECTED CHICK EMBRYOS. *Abram B. Stavitsky and Henry F. Lee*, California Institute of Technology, Pasadena 4, California.

A new technique for intravenous injection of chick embryos made possible the production of disseminated tuberculous lesions within the parenchymatous organs of the embryos. The bacteriostatic effects of compounds may be tested in a short period of time in the presence of intact tissues. Studies utilizing streptomycin and certain other chemotherapeutic substances served to demonstrate the validity of the method. Streptomycin prevented the development of histologic evidence of infection in embryos intravenously inoculated with human tubercle bacilli, but bacilli were recovered upon culturing the tissues.

## THE THEOBALD SMITH SOCIETY

### (NEW JERSEY BRANCH)

NEW BRUNSWICK, NEW JERSEY, JUNE 21, 1947

THE ANTIBACTERIAL PROPERTIES OF PHENOXY-ETHYL-DIMETHYL-DODECYLAMMONIUM BROMIDE (PDDB). *P. C. Eisman and R. L. Mayer*, Research Laboratories, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

A new quarternary ammonium, which possesses strong antibacterial properties, is described. (1) Dilutions ranging between 1:24,000 and 1:768,000 exerted bacteriostatic and fungistatic activity, according to the organism tested. (2) The phenol

coefficient when determined against *Eberthella typhosa* is 433, and when determined against *Staphylococcus aureus* 521. (3) Serum was found to depress activity. (4) Changes in the pH of PDDB solutions greatly influence the killing action against *S. aureus*. (5) A study of the sterilization of surgical instruments was made on scalpel blades contaminated with various organisms including anthrax spores, together with either pus or blood, according to a method described by Spaulding. Complete

sterilization of freshly contaminated blades was obtained with an aqueous solution of 1:1,000 PDDB in less than one-half minute, but 10 minutes were required, depending upon the organism tested, when the contaminants were allowed to dry on the blades before testing. (6) Skin disinfection was tested according to Gardner's technique. Complete sterilization of the test organism (*S. aureus* and *Aerobacter aerogenes*) was obtained in less than 5 minutes.

POTENTIATION OF THE CURATIVE ACTION OF ANTIMALARIAL AGENTS, WITH SPECIAL REFERENCE TO 8-AMINOQUINOLINES AND NAPHTHOQUINONES. *Harry A. Walker*, Division of Pharmacology, Squibb Institute for Medical Research, New Brunswick, New Jersey.

We have reported earlier in periodic malaria reports to the National Research Council that the 8-aminoquinolines, pamaquine and pentaquine, and a naphthoquinone identified as SN-12,230 individually possessed definite curative activity against

*Plasmodium cathemerium* infections in the duck. The amount of drug necessary to demonstrate this action was found to be approximately equal to the maximum tolerated level. However, when pamaquine (or pentaquine) was incorporated into the diet together with SN-12,320, a significant potentiation of the curative action was observed. For example, all animals were cured (100 per cent curative effect) on a diet containing  $\frac{1}{3}$  of the dose of pamaquine which produces a 100 per cent curative effect together with  $\frac{1}{3}$  of the 100 per cent curative dose of SN-12,320. This constitutes a 4-fold potentiation.

As high as an 8-fold potentiation has been obtained with combinations of these 8-aminoquinolines and this naphthoquinone. Potentiation was also observed by combinations of pamaquine or pentaquine with two other naphthoquinones, identified as SN-13,936 and SN-5,949. As yet we have been unable to demonstrate any potentiation of the suppressive activity with combination of these drugs.



# THE EFFECT OF STREPTOMYCIN ON THE METABOLISM OF BENZOIC ACID BY CERTAIN MYCOBACTERIA<sup>1</sup>

ROBERT J. FITZGERALD AND FREDERICK BERNHEIM

*Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*

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Benzoic acid and some related compounds are able to increase the metabolism of mycobacteria (Bernheim, 1941). The oxygen uptake and carbon dioxide production of the pathogenic strains H-37 and B<sub>1</sub> are greatly accelerated when small amounts of benzoic or salicylic (*o*-hydroxybenzoic) acid are added to the bacterial suspensions. Other hydroxybenzoic acids as well as aminobenzoic acids are without effect. Benzoic and salicylic acid are not oxidized by these pathogenic strains, so presumably they act to catalyze some endogenous metabolic reaction of the cell. A *Mycobacterium* obtained from the collection of Dr. Van Niel, recently identified as *M. lacticola*, oxidizes benzoic, *m*- and *p*-hydroxybenzoic acids but not salicylic acid (Bernheim, 1942). The first three acids can act as the sole carbon sources for the growth of this species (Saz and Bernheim, 1942). The metabolism of a number of other mycobacteria with respect to these compounds may be said to be intermediate between that of *M. lacticola* and the pathogenic strains in the sense that benzoic acid is oxidized by them but the hydroxy acids are not, although some of the latter may increase the oxygen uptake. Lehmann (1946) has found essentially the same effects in the mycobacteria he has studied. Because of the apparent importance of benzoic acid in the metabolism of these organisms, it was of interest to study the effects of streptomycin on its oxidation.

## EXPERIMENTAL

*Cultures.* The following nonpathogenic strains of *Mycobacterium* obtainable from the American Type Culture Collection were used: *M. stercoris* (77), *M. avium* (9077), *M. tuberculosis* var. *bovis* (BCG 8240), *M. tuberculosis* var. *bovis* (599), *M. leprae* (4244), and *M. tuberculosis* (607). All of these strains attain full growth in 3 days at 37 C on Long's synthetic medium and thus grow more rapidly than their freshly isolated virulent counterparts.

The H37 and B<sub>1</sub> strains of *M. tuberculosis* were originally obtained from Saranac Lake and have been maintained in these laboratories for several years.

*Media.* *M. lacticola* and *M. stercoris* were grown at room temperature on the medium described by Kohn and Harris (1941) in which glucose is the sole carbon source. For special tests the glucose was replaced by 0.1 per cent benzoic acid or *m*-hydroxybenzoic acid.

<sup>1</sup> A preliminary report has appeared in *Science*, **105**, 435 (1947) and in the Abstracts of Proceedings, 47th General Meeting of the Society of American Bacteriologists, 68 (1947). This study was aided in part by a grant from the Duke University Research Council.

The pathogenic H37 and B<sub>1</sub> strains were grown on veal infusion glycerol broth, whereas the other mycobacteria were grown on Long's synthetic medium at 37 C. For special tests the following modifications of the medium described by Dubos *et al.* (1946) were used.

Asparagine (or benzoic acid).....	1.0 g
Ammonium citrate.....	1.0 g
KH <sub>2</sub> PO <sub>4</sub> ·12H <sub>2</sub> O.....	1.0 g
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O.....	6.3 g
Ferric ammonium citrate.....	0.1 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.6 g
H <sub>2</sub> O.....	to 1,000.0 ml

The pH was adjusted to 7.0. This medium was also used with the addition of 2.0 per cent agar.

Cultures were used when they were at the beginning or middle of the logarithmic growth phase. Even suspensions of washed bacteria were made by the method already described (Bernheim, 1941). An aliquot (3 to 7 mg dry weight of bacteria) in 2.0 ml of M 20 phosphate buffer pH 6.7 was placed in each Warburg vessel and the oxygen uptake measured with and without the addition of various compounds. Streptomycin HCl (Merck) was used. It was made up in saline and diluted with the phosphate buffer immediately before its addition to the suspension. All experiments were done at 37 C in air.

The first experiments showed that the oxidation of benzoic acid by most of these bacteria is markedly sensitive to streptomycin. As shown in figure 1, 1.0 µg in 2.0 ml causes an appreciable inhibition, and with 10.0 µg in 2.0 ml the inhibition is almost complete. Figure 1 also shows that 500 µg of streptamine SO<sub>4</sub> or streptidine HCl (kindly supplied by Merck and Company) are without effect. Apparently the whole streptomycin molecule is necessary for the inhibition of the oxidation of benzoic acid, as it is for the inhibition of growth. Streptomycin, however, has no effect on the metabolism of the pathogenic bacteria which do not oxidize benzoic acid. Even 500 µg have no effect on the increased uptake in the presence of benzoic and salicylic acids and have little effect on the oxidation of fatty acids and other substrates. Pathogenicity in these mycobacteria is accompanied by a change in metabolism and a greatly decreased sensitivity of oxidative reactions to streptomycin. This difference is not reflected in the effects of streptomycin on the growth of these organisms.

It was then necessary to determine whether the oxidation of benzoic acid was the reaction most sensitive to streptomycin. Various possible substrates were added to suspensions of the bacteria. No amino acid tested is oxidized with the exception of tyrosine by *M. lacticola* and asparagine by some of the other species. Higher and lower fatty acids are oxidized rapidly by all of them. Trehalose is the only sugar which is readily oxidized by most of the strains, the exceptions being the pathogenic H37 and *M. stercoris*. Trehalose is a disaccharide (α-glucosido-1-α-glucoside) containing two molecules of glucose linked through their aldehyde groups. Other glucose disaccharides, such as cellobiose and maltose as

well as glucose itself, that have free reducing groups are not oxidized. The exception again is *M. lacticola*, which apparently shows much less specificity than any of the other species. Fructose is oxidized slowly by most of the organisms, as are mucic acid, mannitol, and sorbitol. These results are shown in table 1, which also indicates which compounds can act as sole sources of carbon for growth. Figures 2 and 3 show the effects of streptomycin on the oxidation of a number of these compounds in comparison with its effects on the oxidation of

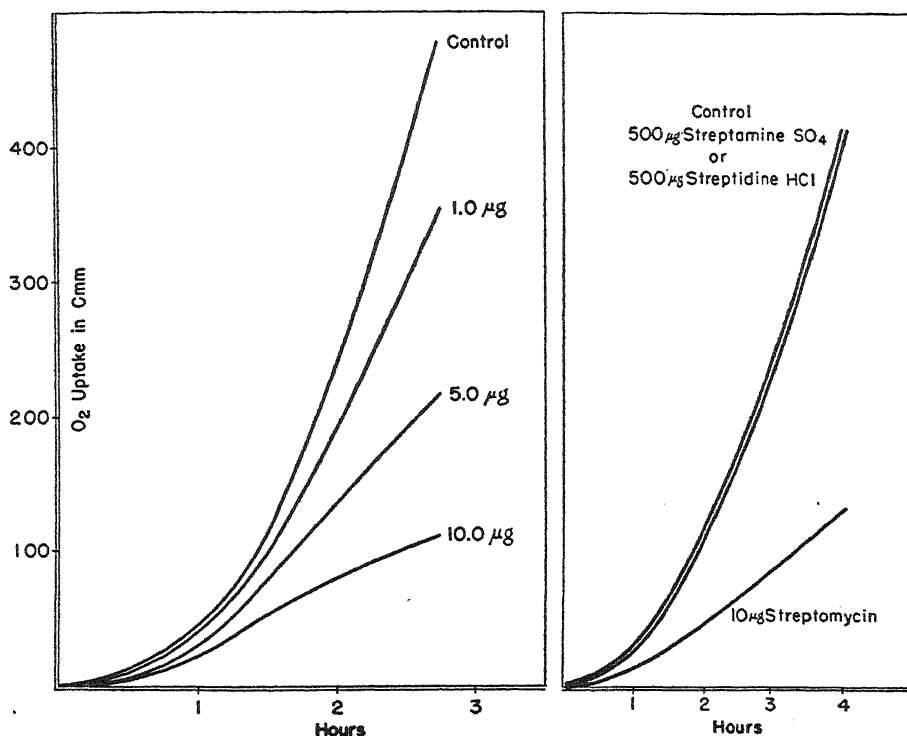


FIG. 1. Left: Effect of different amounts of streptomycin on the oxidation of benzoic acid by *M. 607*. Right: The effect of streptomycin, streptamine, and streptidine on the oxidation of benzoic acid by *M. BCG*.

benzoic acid. In all cases the oxidation of benzoic acid is inhibited to a greater extent than that of any other compound tested. *M. lacticola* oxidized *m*- and *p*-hydroxybenzoic acids as well as phenol and tyrosine. The oxidation of these hydroxy compounds is somewhat more sensitive to streptomycin than that of benzoic acid, but all these reactions require 50 µg per ml of streptomycin for inhibitions comparable to those obtained by 5.0 µg per ml in all the other non-pathogenic species.

The next question which arose was whether organisms made resistant to streptomycin by growing them in gradually increasing concentrations of the drug would show a corresponding resistance to the effect of this drug on the oxidation

of benzoic acid. These experiments were done on *M. lacticola* and *Mycobacterium* 607. The results are shown in table 2 and indicate that a parallelism exists between the ability of these bacteria to grow in streptomycin and their ability to oxidize benzoic acid in its presence. Since streptomycin-resistant organisms did not inactivate the drug, it is possible to assume provisionally that resistance to it is accompanied by the production of more of the catalyst or catalysts responsible for the oxidation of benzoic acid. Support is given to this assumption by the following experiments. *M. lacticola* was grown in a medium with benzoic acid as the sole carbon source. The oxidation of benzoic acid by these organisms was not inhibited by 300  $\mu$ g per ml streptomycin, whereas the oxidation by the control grown in glucose was inhibited as usual by 50  $\mu$ g per ml. The oxidation of *p*-hydroxybenzoic acid by the benzoate strain was as sensitive to streptomycin as the oxidation of this compound by the control. This proves, incidentally, that the oxidation of benzoic acid by *M. lacticola* does not go through the hy-

TABLE 1  
*Properties of the cultures*

ORGANISM	BENZOIC ACID	SALICYLIC ACID	<i>p</i> -HY- DROXY- BENZOIC ACID	TREHA- LOSE	FRUCTOSE	GLUCOSE	MUCIC ACID	ACETIC ACID	PALMITIC ACID	MANNI- TOL
<i>M. leprae</i> ...	+ C	—	—	+			—		+	+
<i>M.</i> 607.....	+ C	— X	— X	+ C	+ C	+ C	+ X	+	+	+ C
<i>M. stercoris</i> ..	+ C	—	—	—	+	— C		+	+	
<i>M. avium</i> ....	+ X	—	—	+		—				
<i>M.</i> BCG....	+ C	—	—	+	+	— C	+	+	+	+
<i>M. phlei</i> ....	—	—	—	+		$\pm$ C			+	+
<i>M.</i> H37.....	+ X	+ X	— X	—	—	— C	—	+	+	—
Soil <i>M.</i> .....	+ C	—	+ C	+	+	+ C	+	+	+	+

+ = increased O<sub>2</sub> uptake; — = no increased O<sub>2</sub> uptake; C = utilized as sole C source; X = not utilized as sole C source.

droxybenzoic acid stage. It also can be shown that when *m*-hydroxybenzoic acid is used as the sole carbon source its oxidation becomes less sensitive to streptomycin, whereas that of benzoic acid has the same sensitivity as the control. Similar experiments were done with *M.* 607, *M.* BCG, and *M. leprae* 4244, grown in modified Dubos medium containing benzoic acid. The results are comparable to those obtained with *M. lacticola*, although the differences are not so great. Table 3 summarizes these facts. It should be noted that in all cases benzoic acid at the beginning is oxidized more rapidly by the strains grown in it than by the controls. Parallel experiments on the effect of streptomycin on the growth of the benzoic acid and the control strains were not so clear-cut. In a given concentration of streptomycin the benzoic acid strains grew out more rapidly for a day or two but were then overtaken by the control strains. It is possible that the benzoic acid strains have lost their ability to alter their metabolism as readily as the controls.

The organisms grown in benzoic acid contain a dark pigment, and their control



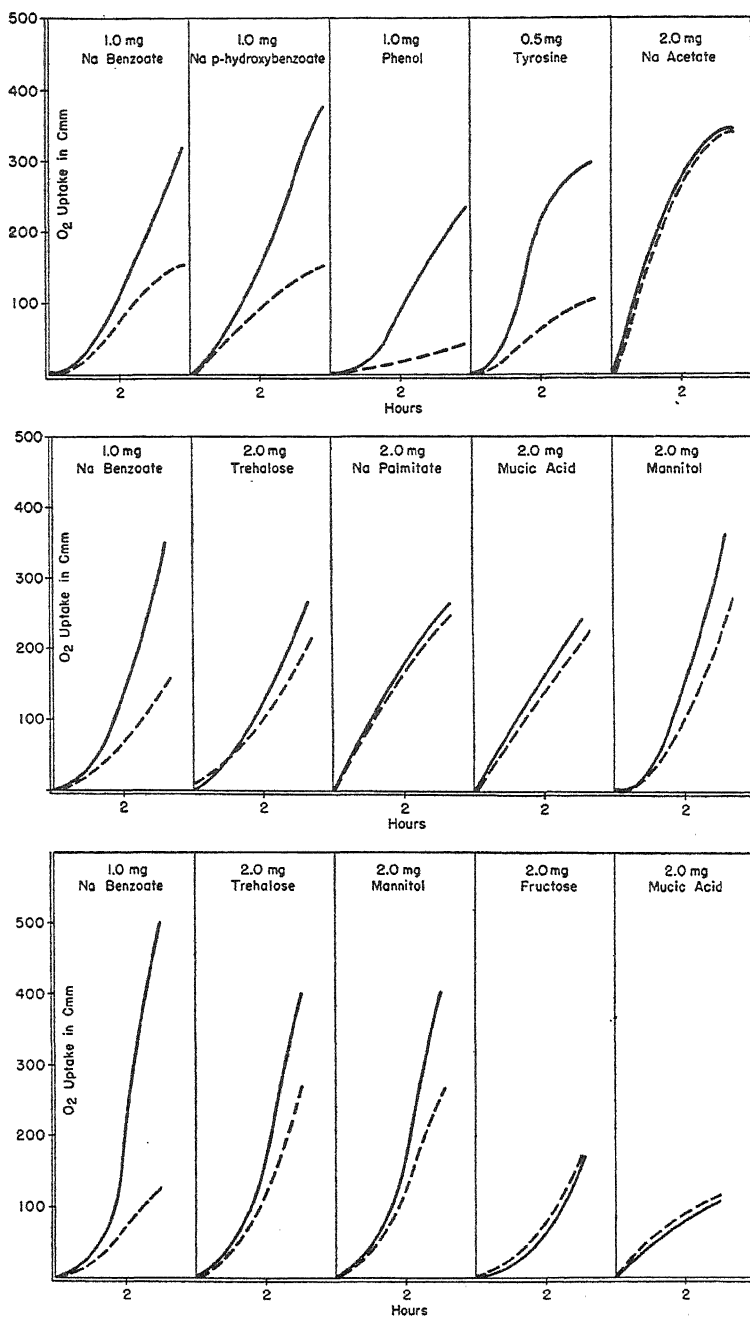


FIG. 2. *Top*: Effect of 100  $\mu$ g per ml of streptomycin on the oxidation of various compounds by the soil mycobacterium. *Center*: Effect of 5  $\mu$ g per ml of streptomycin on the oxidation of various compounds by *M. BCG*. *Bottom*: Effect of 5  $\mu$ g per ml of streptomycin on the oxidation of various compounds by *M. 607*.

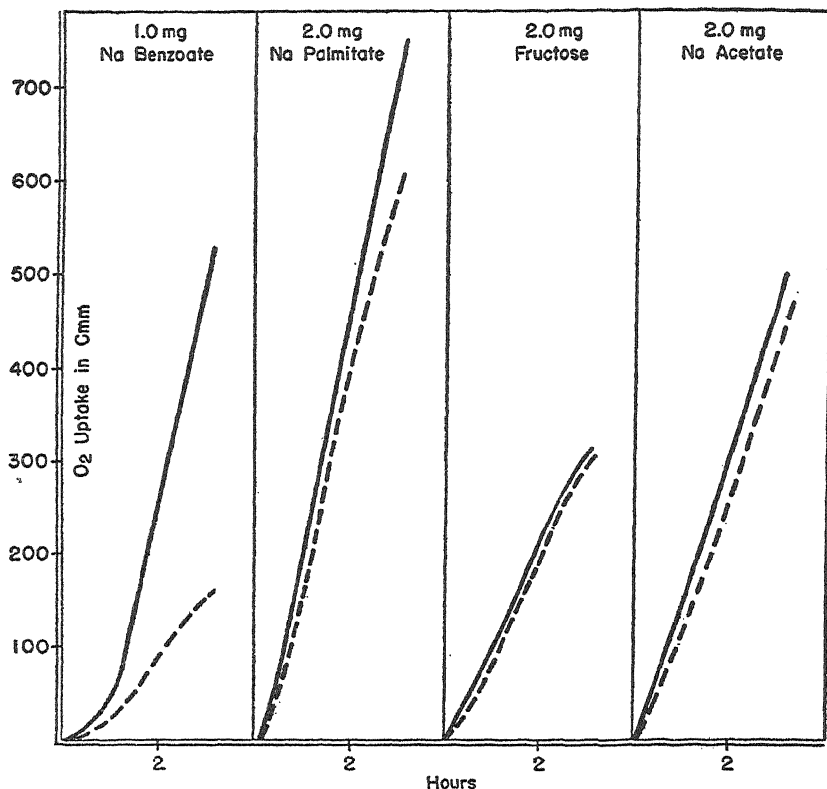


FIG. 3. EFFECT OF 5 µg PER ML OF STREPTOMYCIN ON THE OXIDATION OF VARIOUS COMPOUNDS BY *M. STERCORIS*

TABLE 2

*Effect of streptomycin on benzoate oxidation of normal and resistant strains of mycobacteria*  
Percentage of Inhibition of Benzoate Oxidation

EXPERIMENT	M. TUBERCULOSIS NO. 607 (5.0 µg/ML STREPTOMYCIN)				
	Normal	R 125 µg %	R 250 µg %	R 500 µg %	R 10,000 µg %
1	64			31	
2	81	90	54	29	
3	85		62		
4	76	59	56	47	
5	74	57	52	26	
6	85				0
	SOIL MYCOBACTERIUM (100 µg/ML STREPTOMYCIN)				
	Normal	R 300 µg %	R 600 µg %	R 1,200 µg %	
1	57	53	11	0	
2	50	25	0	0	
3	64	61	19	16	
4	60	53	22		

or resting respiration is always less than the controls, possibly because they have stored up less oxidizable material in their cells. It should be emphasized, however, that in comparing the oxidation of the benzoic acid and control strains the same amounts of bacteria were always used. This is important because the percentage of inhibition by streptomycin is proportional to the number of organisms present. Thus with *M. 607* a 29 per cent inhibition of the oxidation of benzoic acid was obtained when 0.5 ml of the bacterial suspension was used, whereas a

TABLE 3

*The inhibition of streptomycin of the oxidation of benzoic and m-hydroxybenzoic acid by various mycobacteria grown with and without benzoic or m-hydroxybenzoic acid in the medium*

(The oxygen uptake of the organisms without added benzoic acid has been subtracted in each case)

M. 607, CONTROL				M. 607 GROWN WITH BENZOATE			M. LEPRAE 4244 CONTROL			M. LEPRAE 4244 GROWN WITH BENZOATE		
Time	Benzoate	Benzoate + 5 µg/ml S	I	Benzoate	Benzoate + 5 µg/ml S	I	Benzoate	Benzoate + 5 µg/ml S	I	Benzoate	Benzoate + 5 µg/ml S	I
hr	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%
0.5	7	10	0	15	15	0	0	2	0	11	10	0
1.0	33	26	21	56	47	16	7	5	28	25	22	12
2.0	156	67	57	165	131	21	30	10	66	42	35	17
3.0	352	107	70	297	222	25	60	15	75	56	48	14
4.0	525	141	73	398	289	29	112	18	84	80	64	20
5.0	539	175	69	493	349	29						

M. LACTICOLA CONTROL							M. LACTICOLA GROWN ON BENZOATE AS SOLE C SOURCE						M. LACTICOLA GROWN WITH m-HYDROXYBENZOATE AS SOLE C SOURCE					
Time	Benzoate	Benzoate + 100 µg/ml S	I	m-Hydroxybenzoate	m-Hydroxybenzoate + 100 µg/ml S	I	Benzoate	Benzoate + 100 µg/ml S	I	m-Hydroxybenzoate	m-Hydroxybenzoate + 100 µg/ml S	I	m-Hydroxybenzoate	m-Hydroxybenzoate + 100 µg/ml S	I			
hr	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%	mm <sup>3</sup>	mm <sup>3</sup>	%			
0.5	0	0	0	0	0	0	27	46	0	15	13	0	55	57	0			
1.0	0	0	0	0	0	0	95	110	0	57	32	44	90	94	0			
2.0	70	29	59	95	55	42	254	239	6	168	66	61	177	186	0			
3.0	163	47	71	187	90	52	378	322	15	242	97	59	317	335	0			
4.0	242	60	75	249	117	53	459	368	20	282	117	59						

I = inhibition; S = streptomycin.

52 per cent inhibition occurred with 0.25 ml. In this connection it may also be mentioned that the resting respiration is never inhibited by streptomycin.

There is always a latent period before the inhibition of the oxidation of benzoic acid takes effect. This may be due to a slow penetration of streptomycin into the cell or to the fact that the drug is not inhibiting the first step in the oxidation of benzoic acid but some intermediate step. Streptomycin was therefore added to the bacterial suspension at different intervals during the oxidation of benzoic

acid, and the time was measured for the attainment of a given percentage of inhibition. The time was the same whether the streptomycin was added before the benzoic acid or halfway through the oxidation process, and this indicates that the latent period is due to the time necessary for the drug to penetrate to its site of action. It also indicates that it is the oxidation of benzoic acid which is inhibited, but does not rule out the possibility that the oxidation of intermediates is also affected.

Attempts were made to determine the effect of benzoic acid concentrations on the inhibition by streptomycin, to see whether the two substances are competing for the enzyme surface. In order to do this effectively it is necessary to use relatively large concentrations of benzoic acid so that its concentration is not materially altered by the amount metabolized. It was not possible to use such concentrations as they tended to be inhibitory. Consequently, the percentage of inhibition was measured when 1.0 and 2.0 mg of benzoic acid were added and a constant amount of streptomycin was used. The first significant figures were taken after the latent period when 20 per cent or less of the benzoic acid had been oxidized. Under these conditions the following percentages of inhibition were obtained for 1.0 and 2.0 mg benzoic acid, respectively, and 5.0  $\mu$ g per ml streptomycin: 38 and 25 per cent for *M. avium*, 25 and 12 per cent for *M. BCG*. As the oxidation proceeds the percentages of inhibition increase and become the same for the two concentrations of benzoic acid. The indication is, therefore, that the substrate and inhibitor are competing for the enzyme.

None of the species completely oxidize benzoic acid to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Usually the oxidation stops when a half of the theoretical amount of oxygen is taken up. The end product has not been identified.

#### DISCUSSION

A bacteriostatic agent may act by inhibiting anabolic reactions directly, thus limiting growth by interfering with the synthesis of cellular material, or indirectly by inhibiting oxidative reactions which provide the energy for such synthesis. It is evident that certain nonpathogenic mycobacteria can utilize benzoic acid as a source of energy and that this reaction is readily inhibited by streptomycin. Since there is, moreover, a parallelism between the ability of the drug to inhibit this oxidation and the growth of the organism, the evidence indicates that this may be an important mechanism in the bacteriostatic action of streptomycin. It is undoubtedly not the only mechanism. The growth of pathogenic mycobacteria which do not oxidize benzoic acid is equally well inhibited by the drug, and this is also true of a number of gram-negative bacteria that do not utilize this compound. It would seem, therefore, that there must be some general reaction which is inhibited in these organisms by streptomycin and that the inhibition of oxidative reactions is secondary. It is of interest, however, that a specific oxidation can be inhibited by a so-called antibiotic agent.

#### SUMMARY

A number of nonpathogenic mycobacteria oxidize benzoic acid. This oxidation is inhibited by very small amounts of streptomycin.

The oxidation by these organisms of trehalose, mannitol, fatty acids, and certain other substances is less sensitive to streptomycin.

As the growth of the organisms becomes resistant to streptomycin, so does the oxidation of benzoic acid.

The oxidation of benzoic acid by mycobacteria grown in benzoic acid as the sole carbon source or grown in media containing benzoic acid is more resistant to the inhibiting action of streptomycin.

Streptamine and streptidine which do not inhibit growth do not inhibit the oxidation of benzoic acid.

Benzoic and salicylic acid stimulate the oxygen uptake of pathogenic mycobacteria but are not oxidized by them. This reaction is not inhibited by streptomycin.

Other properties of the action of streptomycin on the oxidation of benzoic acid are described.

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## CONCERNING FLAGELLATION AND MOTILITY

H. J. CONN AND R. P. ELROD

New York Agricultural Experiment Station, Geneva, New York,<sup>1</sup> and Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, New Jersey

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In certain recent papers Pijper (1946, 1947) has proposed the thesis that bacterial flagella are not organs of locomotion but are artifacts produced on the drying of a carbohydrate envelope surrounding the cell. He regards this as the explanation of much disagreement which has occurred in the literature about the type of flagellation shown by any given species and insists that since flagella are mere artifacts their arrangement around the cell is of no significance. Some time ago one of the present authors (Conn, 1938) proposed an entirely different theory to account for the bacterial species that were declared peritrichic by some students, monotrichic by others; namely, that some species are neither constantly peritrichic nor truly monotrichic, but that they show "degenerate peritrichic flagellation," some strains having only one flagellum, others two or three flagella, but never a tuft or two or three at one pole.

The present investigation was undertaken partly in the hope that the electron microscope would shed some light on the subject and partly with the idea of using Pijper's technique on a different motile organism.

### CULTURES SELECTED FOR STUDY

When the idea of degenerate peritrichic flagellation was advanced, it was indicated as being especially well represented by species of the genera *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. Accordingly, for the present electron microscope study representatives of these genera were selected as follows: *Agrobacterium tumefaciens* (Smith and Townsend) Conn (the type species of the genus); *Agrobacterium radiobacter* (Beijerinck and Van Delden) Conn; *Agrobacterium rhizogenes* (Riker *et al.*) Conn; strains of *Rhizobium* from pea, clover, and alfalfa nodules; two strains of *Chromobacterium* spp. (violet bacteria, Cruess-Callaghan's nos. 17 and 19).

As organisms for use in trying the Pijper technique, *Escherichia coli* and *Bacillus cereus* were selected.

### TECHNIQUE FOR PREPARING MOUNTS FOR THE ELECTRON MICROSCOPE

It has been our experience, and that of others, that any excessive manipulation of bacterial material in preparing mounts for the electron microscope invariably results in mutilation. Therefore, although a few of the pictures represented here were taken of organisms prepared in the usual way (distilled water preparations

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dried on collodion mounted on screens), the majority have been the result of a stripping technique.

Williams and Wyckoff (1946) and Schaeffer and Harker (1942) have described techniques for the preparation of true replicas for observation in the electron microscope. Recently Hillier and Baker (1946) have described a technique wherein the top layer of organisms from a young colony was removed in place of an expected replica. In the course of this study hundreds of mounts prepared by the ordinary method were made. In only a few instances have we been able to observe organisms with intact flagella.

The method employed in the present work was as follows: The organisms were grown on fresh lima bean agar slopes or agar plates for about 16 hours. A clean microscope slide was then either touched to the growth on the plates and a drop of distilled water added to the adhering organisms, or a loopful of material was transferred to a drop of distilled water on the slide. Bacteria and water were then allowed to stand for 30 minutes. This part of the procedure approaches that recently described by Knaysi *et al.* (1947). At the end of 30 minutes additional distilled water, enough to flood the slide, was added. The slide was gently rotated a few times and the water poured off, the slide drying in a vertical position.

After thoroughly drying, the slides were then shadow-casted with gold, about 8 to 10 Å of gold being deposited on the slide at an angle of 15 degrees. A solution of 0.5 per cent collodion in amyl acetate was allowed to run over the slide, and the slides were again dried in a vertical position. This collodion film was then floated off and mounted on the screens in the usual fashion.

Although we expected to obtain shadow-casted replicas, this did not prevail. Instead, the organisms and gold film were picked up *in toto* by the collodion. This did not obviate but rather enhanced our preparations. In almost every instance the organisms were observed with intact flagella. Even the slight manipulation employed, however, resulted in detaching some flagella, which could be observed in many fields. The pictures utilized represent organisms that had in most instances no detached flagella in the near vicinity.

An RCA type EMC-1 electron microscope was employed.

#### FLAGELLATION OF AGROBACTERIUM SPECIES

The first work on flagellation of *A. tumefaciens* (crown gall organism) done with the electron microscope, part of which has already been published (Braun and Elrod, 1946), seemed to indicate clearly a single polar flagellum (figure 1, no. 1). Later it came to be noticed that the flagellum was not always attached exactly at the pole (figure 1, nos. 2, 4, and 5), a circumstance previously stated (Conn, Wolfe, and Ford, 1940) to indicate degenerate peritrichic flagellation.

Preparations made at about the same time from *A. rhizogenes* (cause of hairy root) and *A. radiobacter* also showed a decided predominance of cells with single flagella, usually at the pole (figure 1, nos. 3, 6). These organisms like *A. tumefaciens* have been described, by some authors at least, as having peritrichous flagella.



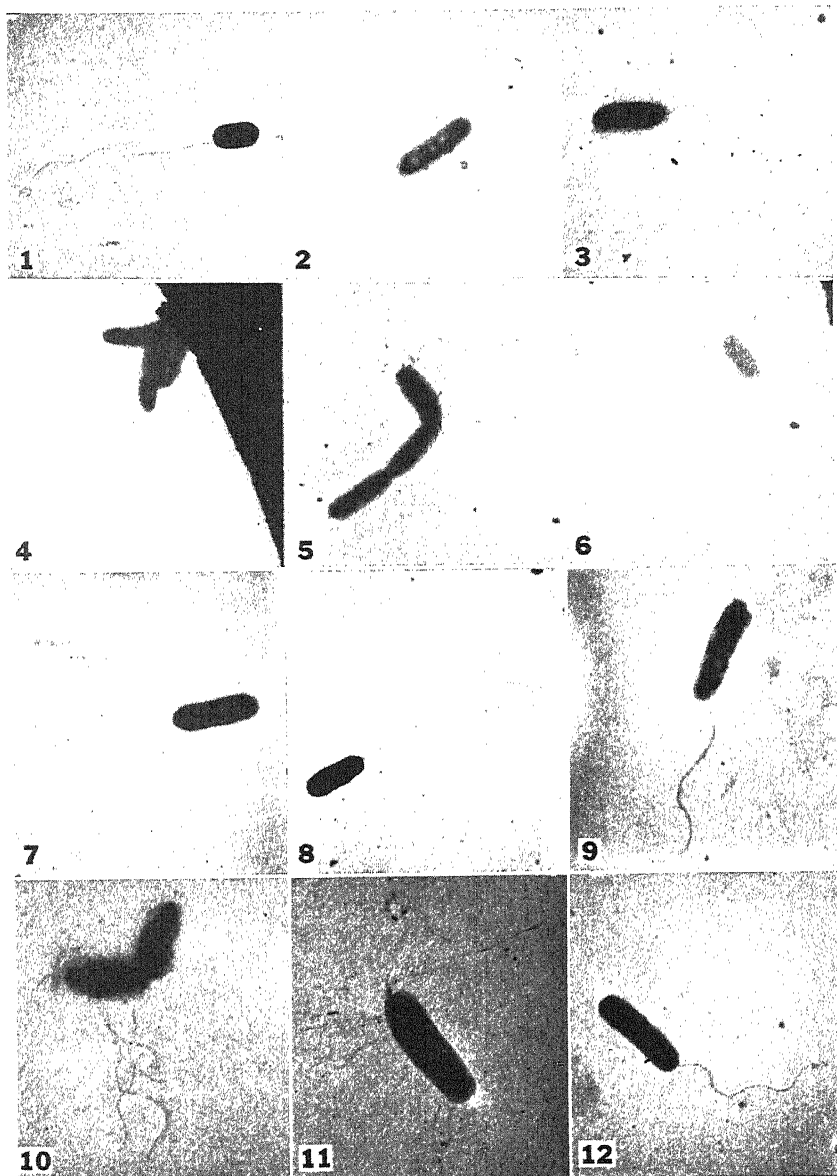


FIG. 1. ELECTRON MICROGRAPHS (GOLD SHADOWED) SHOWING FLAGELLA  
 Nos. 1, 2, 4, and 5. *Agrobacterium tumefaciens*.  
 No. 3. *Agrobacterium rhizogenes*.  
 No. 6. *Agrobacterium radiobacter*.  
 Nos. 7 and 8. Cruess-Callaghan's culture no. 19, "*Bacillus violaceus*."  
 No. 9. Cruess-Callaghan's culture no. 17, "*Bacillus membranaceus amethysticus*."  
 No. 10. *Rhizobium* culture from pea nodule.  
 No. 11. *Rhizobium* from clover nodule.  
 No. 12. *Rhizobium* from alfalfa nodule.

As the same situation was found in the case of the violet *Chromobacterium* species (figure 1, nos. 7, 8, 9), it was decided to make a more intensive study of certain other organisms (the legume nodule bacteria) thought to have the same type of flagellation.

#### FLAGELLATION OF RHIZOBIUM

The first micrographs of *Rhizobium* species were made on single strains of the clover, pea, and alfalfa nodule organisms and are shown in figure 1, nos. 10 to 12. These micrographs were made after numerous fields were examined visually and are regarded as entirely typical of the majority of organisms present. The impression to be gained from these preparations is clearly that the clover and alfalfa cultures each have a single polar flagellum, whereas the pea organism may be peritrichic. Now these species belong to the group of nodule bacteria which are generally recognized as peritrichic (the soybean and the cowpea organisms, on the other hand, being regarded as monotrichic). The alfalfa organism, in particular, has so generally been accepted as peritrichic, and so many photomicrographs indicating as much have been published, that these results seemed to call for further study.

Accordingly a collection of 12 strains of the alfalfa organism was obtained from Dr. A. W. Hofer of Geneva, New York, and they were studied by essentially the same technique. Electron micrographs (one of each strain) are shown in figure 2. It is clearly seen that some cells are monotrichic, others peritrichic. In every instance typical cells were selected, after considerable search of each preparation, before the micrograph was made. In other words, some strains seemed to show one type of flagellation, others the other; that is, there are both monotrichic and peritrichic strains of this species.

Apparently, therefore, the electron microscope bears out in regard to the *Rhizobium* species the same conclusion that had been drawn from stained preparations, namely, that peritrichic and monotrichic strains may occur in the same species, probably monotrichic and peritrichic cells in the same strain. Obviously, in the case of such organisms as this, the type of flagellation cannot be employed as a criterion for species diagnosis. This conclusion does not, however, invalidate type of flagellation as a diagnostic feature in the case of truly peritrichic species (as in the colon-typhoid group) or definitely lophotrichic forms (like *Pseudomonas fluorescens*).

Present work with the electron microscope on the other organisms discussed above has not included a sufficient number of strains to show whether the same conclusion could be drawn concerning them. The presumption is that such would be the case. If that is true, it seems logical enough to explain observed discrepancies on the basis of degenerate peritrichic flagellation.

#### SIGNIFICANCE OF FLAGELLATION

Pijper's explanation of such discrepancies as due to flagella being artifacts not concerned in motility seems harder to accept. There are several arguments against this theory:

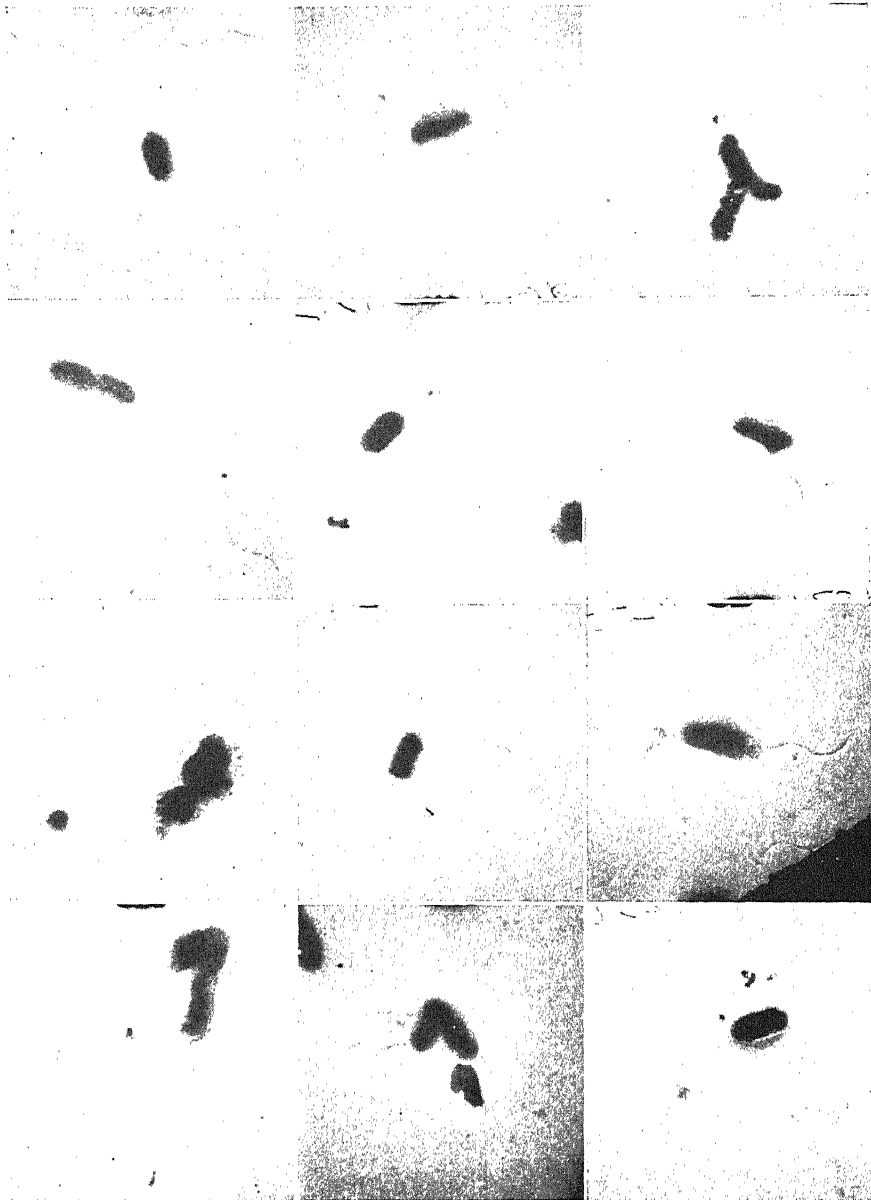


FIG. 2. ELECTRON MICROGRAPHS (GOLD SHADOWED) SHOWING FLAGELLATION OF 12 STRAINS OF RHIZOBIUM FROM ALFALFA NODULES  
Each micrograph was taken from a field typical of that particular strain.

(1) Electron micrographs do not, as he claims, indicate flagella to be merely part of the capsular material, but rather suggest that they are definite entities attached to the very body of the cell.

(2) The writers have been privileged to see an electron micrograph (no. 2011) of *Vibrio metchnikovii*, taken by Miss vanIterson in Dr. Kluyver's laboratory at Delft, Holland (Netherlands Institute for Electron Microscopy), which is to be published shortly in *Biochimica et Biophysica Acta*. This micrograph of a shadow-casted specimen shows a monotrichic organism with its flagellum attached to a protuberance arising not from the cell wall but from the body of the cell itself; in the instances shown in the micrograph the attachment is either slightly below or above the mid-line of the cell. In one instance the flagellum has been torn off, and the rupture in the cell wall is evident. The preparation is unusually clear, and the flagellum is so regular and substantial in its entire aspect that it cannot be conceived as an artifact.

(3) The electron microscope indicates the true shape of cells better than stained preparations and shows many of the motile bacteria to be too short to move through the medium by an undulatory motion.

(4) A motile sarcina was described by Burri many years ago and has been observed by each of the authors. In this species a packet of eight cells swims as a unit. Such a group of cells certainly cannot move by means of the mechanism described by Pijper. The same applies to other motile cocci.

(5) Finally, studies of *Bacillus cereus* and *Escherichia coli* have been made by one of the authors in "methocel" solutions of various concentrations, as recommended by Pijper, and these have been studied under dark-field illumination. This technique brings out several of the effects noted by Pijper. It slows down the motility and enables the observer to note the rotation of the cells around their longitudinal axis while moving through the medium. With the organisms investigated, however, only a few of the cells appear to be flexible; the individual cells are often short and appear perfectly rigid. Nevertheless, the rigid cells are just as motile as those which undulate. No "tails" whatsoever have been observed.

The writers do not question Pijper's observations on the typhoid organisms but merely the validity of drawing general conclusions therefrom as to the mechanism of locomotion of bacteria in general. It is obvious that if other bacteria can move through the same type of medium without flexing, his explanation of locomotion cannot apply to all bacteria. It may well be true that some species are surrounded by capsular material which trails behind them when they are swimming; but this observation does not exclude the possibility of their having flagella also, which are too small to be seen while in motion, even by use of brilliant dark-field illumination.

Accordingly, in the writers' opinion flagella must still be regarded as having the significance attached to them in the past. One must, apparently, use considerable caution in describing any organism as either peritrichic or monotrichic, since there seem to be species in which the peritrichic flagellation is of a degenerate type, so that strains with only a single flagellum to the cell can be found. There are, nevertheless, some species that are uniformly peritrichic, others uniformly lophotrichic, and others with a single flagellum which is always attached at the pole. Careful study of the numerous strains should always show which type of flagellation a given species possesses, but diagnosis of flagellation without such study is often misleading.

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# AN IMPROVED LABORATORY-SCALE FERMENTOR FOR SUBMERGED CULTURE INVESTIGATIONS

HARRY HUMFELD

*Western Regional Research Laboratory,<sup>1</sup> Albany, California*

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A small laboratory fermentor which employs mechanical agitation for the dispersion of air introduced under pressure has previously been described by Feustel and Humfeld (1946). This fermentor has an operating capacity of 500 to 2,000 ml and has been found very useful for small-scale, yeast-culturing investigations, as well as for studies on the production of subtilin activity by *Bacillus subtilis* (1947).

Certain limitations, however, principally those of capacity and effectiveness of foam breaking, have led to the development of an improved fermentor having a larger capacity and a more effective mechanical foam breaker. This fermentor also has a stirring device, which is so designed that air for the aeration of the culture liquid can be drawn in from the atmosphere by suction created behind the stirring blades, as compared with air introduced under pressure. This paper describes the improved fermentor and some of the preliminary results obtained.

## DESCRIPTION OF THE FERMENTOR

The fermentor vessel consists of a standard pyrex glass jar, 12 inches in diameter and 24 inches high. This jar is fitted with a gasketed, stainless-steel cover. The stainless-steel agitation-aeration assembly shown in figure 1 is suspended from the cover and is inserted in the pyrex jar.

Agitation and aeration are accomplished by means of a special air-dispersing device, mounted at the lower end of the stirring shaft near the bottom of the fermentor. Two adjustable truncated cones  $3\frac{1}{2}$  inches in diameter are mounted on the shaft above the agitation-aeration device. By inverting these cones the stirring characteristics may be changed. Four metal struts are attached at right angles to the cover at equidistant points around the periphery of the cover, approximately 1 inch from the edge. A metal web fastened to the lower end of these struts furnishes rigidity and support for the stirring shaft.

The agitation-aeration device consists of a short, central, hollow cylinder, to which four sets of tubes are fastened. These tubes are bent in the form of arcs at right angles to the cylinder. A small vane is attached to each alternate set of tubes. This device rotates between two plates. The upper plate is attached to the supporting web described above. The lower plate is attached to, and kept parallel with, the upper plate by studs. The lower plate has a large circular hole, through which the culture liquid enters. An air-intake pipe extends from the cover to a point directly below the hollow, central core of the agitation-

<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

aeration device. As the turbine device rotates, the suction created behind the vanes at the end of the tubes draws air through the air-intake pipe into the central, hollow cylinder, from which it is conducted into the culture liquid by the radiating tubes. The height of the intake pipe is adjustable at the cover, so that the amount of air drawn in can be regulated. The maximum amount of air is drawn in with the lower opening of the intake pipe raised as high as possible without touching the revolving hollow cylinder of the turbine. Lowering the intake pipe decreases the air flow.

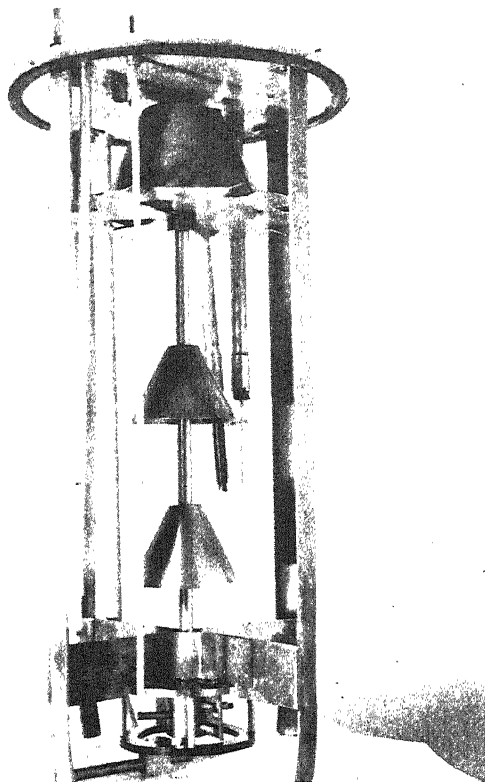


FIG. 1. FERMENTOR ASSEMBLY FOR LARGE LABORATORY FERMENTOR

The foam-breaking device consists essentially of a disk mounted on the shaft just below a larger fixed cone. The disk, about 8 inches in diameter, is provided with an interior set of vanes and an exterior set of vanes; half of the vanes of each set are turned up and half are turned down. The outer edge of the cone extends just beyond the interior set of vanes. The inner set of vanes scoops up the foam as it rises to the height of the disk; the centrifugal force created by the rotation throws the foam against the inner surface of the cone, which, in turn, forces it out onto the upper surface of the disk, whose outer set of vanes dis-



tributes the liquid phase against the sides of the fermentor vessel. During this operation the foam is broken effectively, and the liquid is returned down the inside wall of the vessel to the culture liquid.

The fermentor is equipped with a set of pH meter electrodes, the leads of which pass through removable waterproof tubes to the pH meter; hence, the pH of the culture may be determined at any instant. A thermometer inserted through a small well provides for the reading of the temperature of the culture at any time. The fermentor is also provided with a sampling tube, by means of which a sample may be drawn periodically for analyses. The power for operation of the fermentor is furnished by a  $\frac{1}{4}$ -hp ball-bearing, variable-speed, electric motor, mounted vertically at the center of the cover and fastened to the stirring shaft by a self-aligning coupling.

The entire fermentor assembly may be taken apart readily for cleaning and replacement and adjustment of parts. It may be assembled without the motor for sterilization. The operating capacity of the fermentor ranges from 10 to 18 liters of culture medium.<sup>2</sup>

#### OPERATION OF THE FERMENTOR

The fermentor has been found suitable for the submerged culturing of aerobic microorganisms. It has been employed in the propagation of yeast, in which case sterilization of the media and of the equipment is not essential, as well as for the production of antibiotics. For the latter purpose the equipment and the culture medium are sterilized, since the maintenance of pure culture is usually essential. Except for the details of sterilization and care necessary for keeping the culture free of contamination, the technique of operation in all cases is essentially identical. The air drawn in is sterilized by passage through a previously sterilized tube loosely packed with glass wool.

The inoculated medium is transferred to the fermentor, the motor is placed in position on the cover, and the motor shaft and stirrer shaft are connected by means of the self-aligning coupling. The motor is started and the amount of air drawn in is regulated by adjusting the height of the air-inlet pipe by means of the adjusting screw on the fermentor cover. The air flow also may be controlled by adjusting the speed of the motor, which, of course, simultaneously changes the rate of stirring. For most operations a ratio of one volume of air per minute per volume of culture medium is satisfactory. By reducing the distance between the lower tip of the inlet tube and the hollow bore of the agitation-aeration unit, volumes of air as high as two and one-half times the volume of the culture medium may be drawn in per minute. As the volume of growth in the medium increases during the fermentation, the viscosity of the culture suspension gradually increases; hence, if it is desired to maintain a uniform rate of air flow, it is necessary to readjust the distance between the core and the inlet pipe. A record of the pH of the medium and its temperature is kept, and if it is desired to control the pH, a suitable amount of base or acid solution is added as may be required.

<sup>2</sup> Detailed engineering drawings for the construction of similar units may be obtained from the Western Regional Research Laboratory, Albany, California.

Since the fermentation usually generates considerable heat, it has been found desirable to place the fermentor in a suitable water bath and to hold the water in the bath at such a temperature as will maintain that desired in the fermentor. By means of the sampling tube samples are withdrawn periodically for determinations of cell volume, nutrient concentrations, and, in the case of the production of antibiotics, for the bioassay purposes. The fermentor in operation is shown in figure 2.

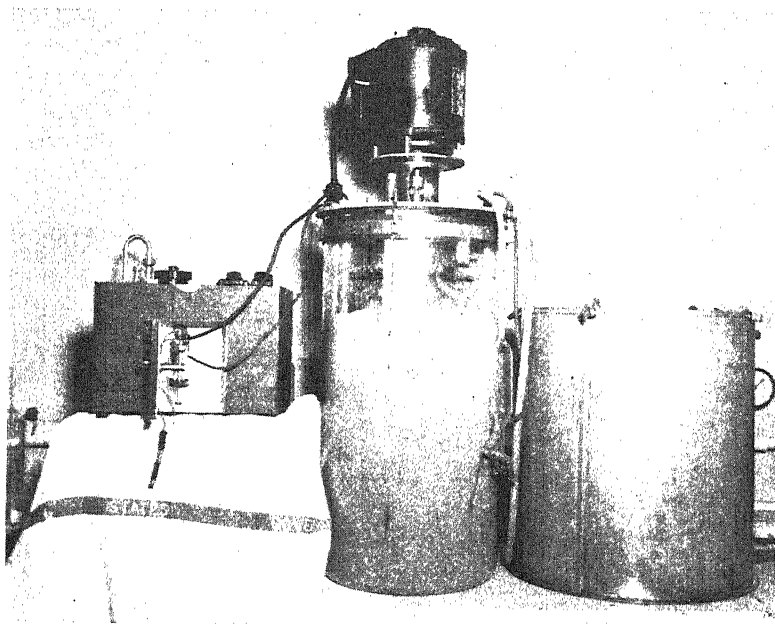


FIG. 2. LARGE LABORATORY FERMENTOR FOR SUBMERGED CULTURE INVESTIGATIONS IN OPERATION

#### FERMENTATIONS

*Yeast production.* An example of the use of the fermentor for yeast production is presented here. The yeast used in this run was *Torulopsis utilis* (NRRL Y-900). The medium was made from pear juice concentrate from cannery pear waste. One liter of the concentrate, which contained 26 per cent sugar, was diluted initially with 12 liters of water. The mineral salts required—15.4 grams of 85 per cent phosphoric acid, 3.6 grams of potassium sulfate, and 1.0 gram of magnesium sulfate—were added.

The inoculum was prepared by transferring a 10-ml suspension from the growth of a stock culture slant to the surface of a shallow layer of wort agar in two Fernbach flasks. The flasks were incubated for 24 hours at 30 C. The growth on the agar was suspended in a small amount of the pear juice medium and, with the rest of the medium, transferred to the fermentor. The motor was operated at

1,160 rpm, and an air volume of 16 liters per minute was used for aeration at the start of the fermentation. Ammonium hydroxide was added to bring the pH to 6.4, and from time to time, as the pH dropped, more ammonia was added.

After the sugar in the pear juice medium at the start of the fermentation had been utilized, additional full-strength concentrate was added from time to time. The details of operation and the yield of yeast obtained are given in table 1.

*Production of antibiotics.* The feasibility of using the fermentor for the production of antibiotics was tested with a culture of *B. subtilis*. The volume of cells and the subtilin activity produced were measured in samples taken periodically during the fermentation run. The fermentor was assembled and sterilized

TABLE 1  
*Results of a yeast propagation experiment*

TIME	pH	YEAST VOLUME	VOLUME IN FERMENTOR	PEAR JUICE CONCENTRATE ADDED	CONC. $\text{NH}_4\text{OH}$ ADDED	AIR	WEIGHT OF YEAST PRODUCED (DRY BASIS)
<i>hr</i>		%	<i>liters</i> 12 $\text{H}_2\text{O}$	<i>liters</i> 1	<i>ml</i> 27	<i>liters/min</i>	<i>g</i>
0	6.4	0.10	13	—	—	16.5	2.9
4	6.2	0.50	13	—	—	16.5	14.3
6	5.0	1.00	13	—	—	16.5	28.6
7½	3.3	3.00	13	—	30	16.5	94.4
9	5.1	5.8	13	—	35	16.5	166
9½	4.2	6.5	13	—	—	16.5	186
10½	3.5	7.5	14	1	55	13.5	231
11½	3.9	10.5	14	—	40	13.5	323
12½	3.6	11.2	15	1	50	11.5	370
13½	5.2	13.0	15	—	25	11.5	429

Total sugar supplied.....	780 g
Dry yeast produced.....	429 g
Yield of yeast (based on sugar supplied).....	55%
Increase of yeast over inoculum.....	148X
Average generation time.....	112 min
Number of generations.....	7.23

in the autoclave for 1 hour at 15 pounds' steam pressure. The procedure was identical with that described by Stubbs *et al.* (1947) for the production of subtilin in small fermentors. The medium was made by diluting 1,400 grams of an asparagus juice concentrate (70 per cent total solids) to 14 liters. This medium was distributed in 3.5-liter portions in 4-liter bottles and sterilized for 30 minutes at 100 C in the autoclave. When cool the reaction of the medium was adjusted aseptically from about pH 5.5 to pH 7 by addition of the required amount of 10 N NaOH, then transferred aseptically to the fermentor, and the inoculum added.

The inoculum was made by suspending the growth from an agar slant in a small amount of medium and adding this suspension to 500-ml portions of the asparagus juice medium in each of two Fernbach flasks. These flasks were incubated

for 24 hours at 35 C; then the contents were transferred to a sterile Waring "blendor," and the bacterial pellicle was thoroughly broken up and suspended in the medium. This suspension was added to the sterile medium in the fermentor.

The initial motor speed was 1,300 rpm, and the aeration was 14 liters of air per minute. As the bacterial cells in the medium multiplied, the medium became more viscous and had a tendency to decrease the rate of aeration. The rpm were gradually increased until 1,600 rpm were attained; thus the rate of aeration was kept uniform. The pH at the start was 6.9 and gradually dropped

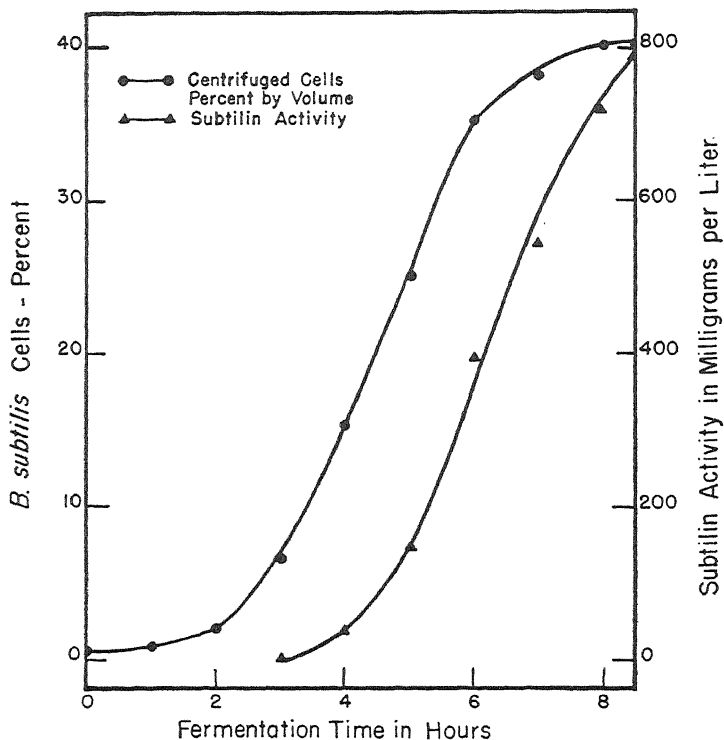


FIG. 3. GROWTH OF *BACILLUS SUBTILIS* AND SUBTILIN PRODUCTION

to 6.15 in 5 hours, after which it gradually increased to 7.45 at 8 hours, when maximum cell volume had been obtained. The culture medium was harvested after 8½ hours' incubation at 35 C.

The bioassay for subtilin activity was made according to the method described by Lewis *et al.* (1947). The volume of the centrifuged cells calculated as percentage of the culture medium and the subtilin activity calculated as milligrams of subtilin per liter are shown in figure 3.

#### DISCUSSION

The evidence presented indicates that this type of fermentor is well suited for yeast production. The size is intermediate between that of the small laboratory

fermentor described by Feustel and Humfeld (1946) and equipment such as might be used in a pilot plant. It is large enough to be used in the investigation of the effects of variations in stirring and aeration, and in foam-breaking devices. Its operation should yield certain information useful in the development of larger, or pilot-plant-scale, fermentors.

The fermentor described in this paper possesses another advantage over the one previously described by Feustel and Humfeld (1946) in that a source of compressed air for aeration is unnecessary. The agitator draws in the air required and disperses it simultaneously. However, the extent to which this manner of aeration might be applicable to larger units is not known.

This fermentor should prove particularly useful in building up inocula in sufficient amounts for pilot plant or commercial operation, as well as for testing variations in the culture medium.

For yeast production it is possible to maintain a high rate of production and a large volume of yeast in the fermentor by periodically withdrawing a portion of the culture whenever the available nutrients have been utilized and then adding fresh medium to replace the amount harvested. In this manner the effect of changing the composition of the medium, rates of agitation and aeration, and other variables may be studied under comparable conditions. Favorable or adverse effects will be reflected in cell volume, rate of growth, or both.

Yields of *T. utilis* that compare favorably with similar results reported in the literature have been obtained. For instance, Feustel and Humfeld (1946) found that in small fermentors the yield of yeast based on sugar supplied was 51.8 per cent, while in the larger fermentor it was 55 per cent. In the small fermentor the increase of yeast over inoculum was 32.8X in 10 hours, while in the larger fermentor it was 148X in 13½ hours. When calculated as average generation time, the figures were 104 and 112 minutes, respectively.

Results obtained in growing *B. subtilis* for subtilin production compare favorably with those obtained by Stubbs *et al.* (1947) in small fermentors. It was found that the growth rate was more rapid in the larger fermentor and resulted in a cell volume of 40 per cent, as compared with a cell volume of about 27 per cent in 8 hours in the small fermentor. The rate of subtilin production, however, was slower in the larger fermentor, especially during the period of 6- to 8-hours' incubation. Since it was not the objective in this study to determine the optimum conditions for maximum subtilin yields, the factors involved were not investigated.

It is evident that the fermentor also lends itself to investigations in pure-culture fermentation, such as the production of antibiotics. In most cases the quantity of available medium will make it possible to obtain data on actual yields of purified material.

#### ACKNOWLEDGMENTS

The author is indebted to Mrs. E. M. Humphreys for making the subtilin assay. Special recognition is accorded Mr. Ernest Aeschlimann and Mr. John R. Hoffman for valuable assistance in the mechanical development and construction of the fermentor.

## SUMMARY AND CONCLUSIONS

A fermentor with an improved agitation-aeration device and a specially designed mechanical foam breaker is described. The agitation-aeration device is so designed as to pull in air for aeration at atmospheric pressure, thus eliminating the use of air under pressure. The foam breaker utilizes a specially designed disk and cone rotated at relatively high speed, which causes the foam to break under the centrifugal force generated under these conditions.

Data are presented which give results of a yeast (*Torulopsis utilis*) propagation run and which indicate a method by which the fermentor may be utilized in continuous operation processes after a constant yeast volume has been attained.

The adaptability of the fermentor for the cultivation of *Bacillus subtilis* for subtilin production has been indicated.

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# CHARACTERISTICS OF *LEUCONOSTOC MESENTEROIDES* FROM CANE JUICE

C. S. McCLESKEY, L. W. FAVILLE, AND REX O. BARNETT

*Louisiana State University, Baton Rouge, Louisiana*

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The bacteria which produce gum or slime in sugar solutions are present in large numbers in the sugar-cane juice of sugar factories and not infrequently cause trouble in the factory processes. These organisms, together with other related forms producing levo-rotatory lactic acid, relatively large amounts of volatile acid, carbon dioxide, and ethyl alcohol from glucose, have been allocated to the genus *Leuconostoc* Van Tieghem by Hucker and Pederson (1930). The species commonly encountered in cane juice is *Leuconostoc mesenteroides* according to the characterization of that species by Hucker and Pederson that was adopted by Bergey *et al.* (1939).

Since this species is known to be rather heterogeneous, both serologically (Hucker, 1932) and biochemically (Hucker and Pederson, 1930), and since this organism has been suggested as a possibility for use in the biological assay of certain amino acids (Dunn *et al.*, 1944; Horn, Jones, and Blum, 1947) and vitamins (Gaines and Stahly, 1943), an investigation of the diversity of strains in this species is desirable. This report is limited to a study of strains isolated from cane juice at the Experimental Sugar Factory of the Louisiana State University.

The older literature pertaining to the organisms of this group has been adequately reviewed by Hucker and Pederson (1930) in their classical work, which demonstrated the essential similarity of variously named cultures isolated from vegetable products, dairy products, and sugar solutions. More recently Niven, Smiley, and Sherman (1941) have pointed out that *Streptococcus salivarius* produces a considerable amount of slime from sucrose, in this way resembling *Leuconostoc*. Also Niven, Kiziuta, and White (1946), White and Niven (1946), and Niven and White (1946) observed that many isolates from cases of subacute bacterial endocarditis produced gum in liquid sucrose media, a characteristic suggestive of the *Leuconostoc* group. The serological relationship of the *Leuconostoc* polysaccharide to that of the type II pneumococcus has been studied by Neill and coworkers (1941).

In this study isolations of *Leuconostoc* strains were made from sugar-house cane juice plated on a medium of the following composition:

Difco tryptone.....	10 g
Difco yeast extract.....	5 g
Raw sugar.....	100 g
Difco agar.....	20 g
Distilled water.....	1,000 ml
pH 6.7. Sterilized 15 minutes at 121 C	

Most of the strains included in this study were isolated from cane juice which had been frozen and stored several months at about  $-18^{\circ}\text{C}$ . The same general types were found also in the fresh juice, but possibly not in the same relative frequency. Care was exercised that all the different colony types of gum formers were isolated for study, and 740 cultures were obtained.

It was noted that there were four distinct types of gum-forming colonies, and the primary grouping of isolates was based on colonial characteristics. These types differed in size, elevation, topography, and optical characters, and were designated A, B, D, and F by Faville (1947). Descriptions of these colonies on

TABLE 1  
*Morphological and colonial characteristics of gum-forming organisms*

MORPHOLOGICAL CHARACTERISTICS (2% RAW SUGAR BROTH)	COLONY TYPES			
	A	B	D	F
Shape.....	Oval, spherical	Oval, spherical	Oval, spherical	Oval, spherical
Size.....	0.5-0.7 by 0.7- 1.0 $\mu$	0.7-0.9 by 0.7- 1.2 $\mu$	0.5-0.7 by 0.7- 1.0 $\mu$	0.5-0.7 by 0.7- 1.0 $\mu$
Grouping.....	Occur in large clusters and pairs; few chains	Occur in pairs and occasional chains of 4-6 cells	Occur in pairs and occasional chains of 4-6 cells	Occur in pairs and occasional long chains of 20-30 cells
COLONIAL CHARACTER- ISTICS (2% RAW SUGAR AGAR)				
Form.....	Circular	Circular	Circular	Circular
Elevation.....	Convex	Conical	Hemispherical	Hemispherical
Height of colony.....	1 mm or less	3-4 mm	4-8 mm	3-4 mm
Diameter of col- ony.....	3-4 mm	4-6 mm	8-12 mm	4-6 mm
Surface.....	Smooth	Rugose	Smooth	Smooth
Margin.....	Entire	Entire	Entire	Entire
Density.....	Semitranspar- ent	Opaque	Transparent	Opaque

10 per cent raw sugar are presented in table 1. Photographs of these types are shown in figures 1 and 2.

The composition of the medium and temperature of incubation had a pronounced effect on the colonial appearance of these cultures. Incubation at 20 to 25  $^{\circ}\text{C}$  on 10 per cent sucrose or raw sugar agar was most satisfactory for showing group differences. When grown at 37  $^{\circ}\text{C}$  colonies of all types were smaller, and there was much less evidence of gum formation. Colonies of types A and D showed little resemblance to those of the same type grown at 20 to 25  $^{\circ}\text{C}$ . At the lower temperature both A and D colonies "dripped" down onto the lid of the inverted petri dish, whereas at 37  $^{\circ}\text{C}$  the colonies were small, opaque, and nearly flat. The colonies were most characteristic after incubation for 3 to 5 days at room temperature. Most strains of the A and D types produced colonies which were so clear that print could be read through them.



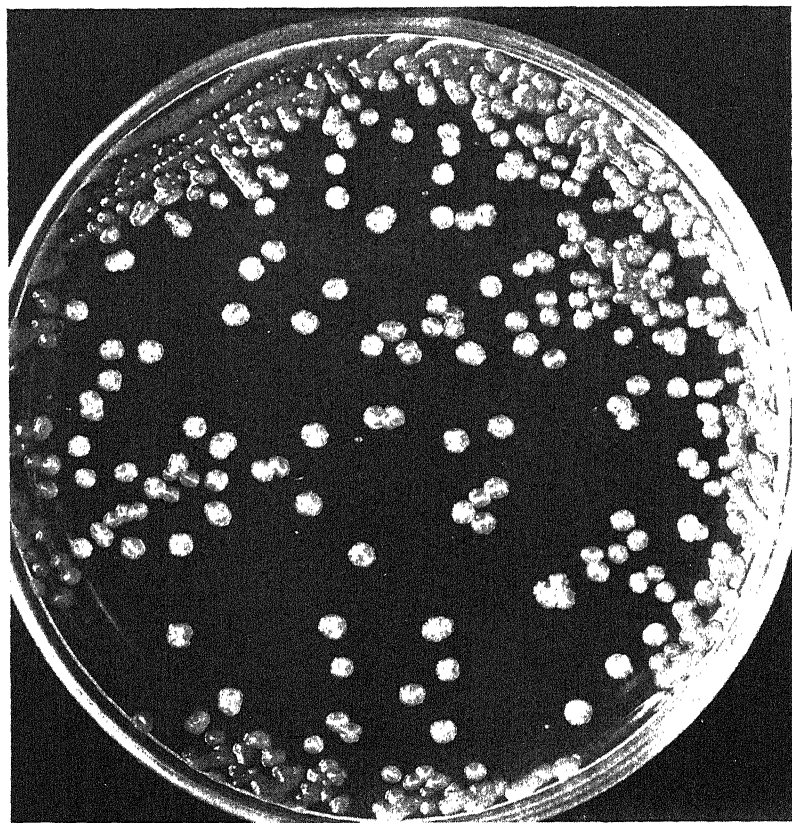
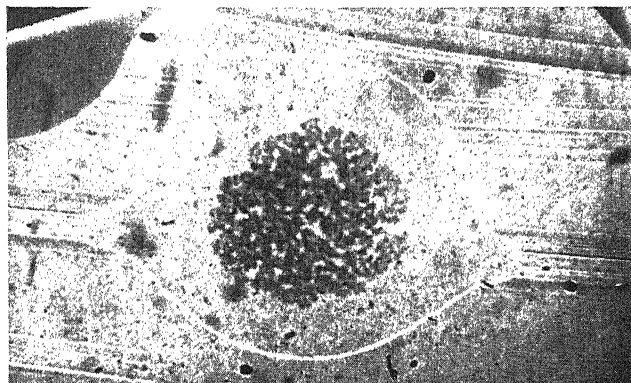


FIG. 1. *Upper*: Strain 730, A type, 1-day culture.  $\times 20$ . *Lower*: Strain 700, B type. Culture on 10% raw sugar agar, incubated at room temperature for 3 days. Colonies are conical, rugose, and cartilaginous. Some strains form a clear, colorless watery gum at the base of the colony.

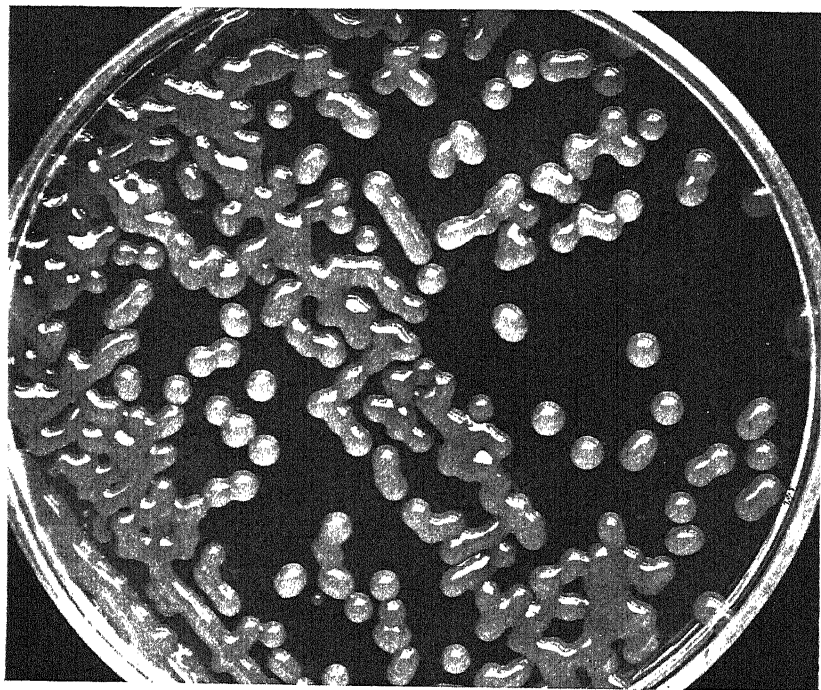
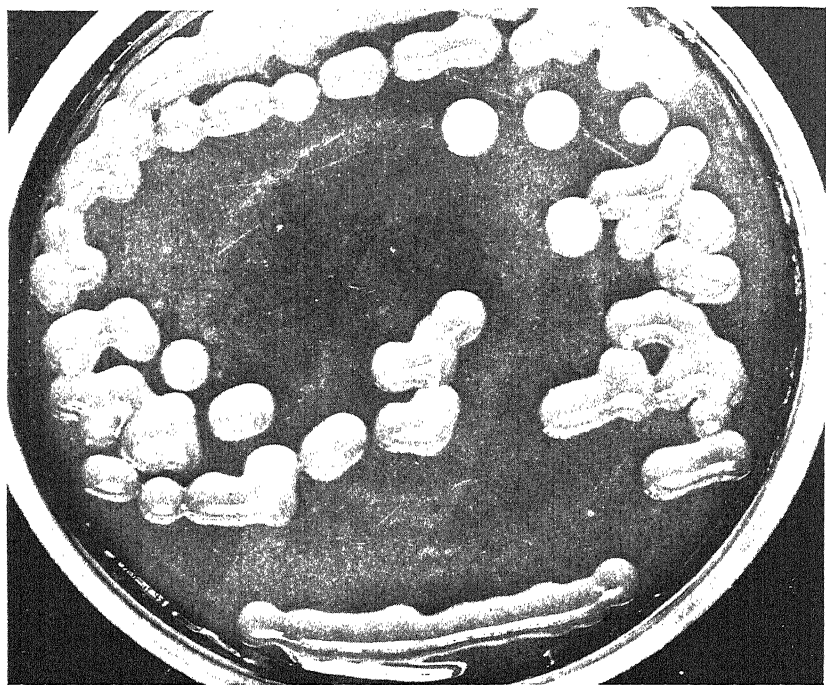


FIG. 2. *Upper*: Strain 200, D type. *Lower*: Strain 860, F type. Three-day culture on 10% raw sugar agar, incubated at room temperature.

There was found to be some correlation between colony type and cell morphology, as indicated in table 1, but morphology alone would not suffice to dis-

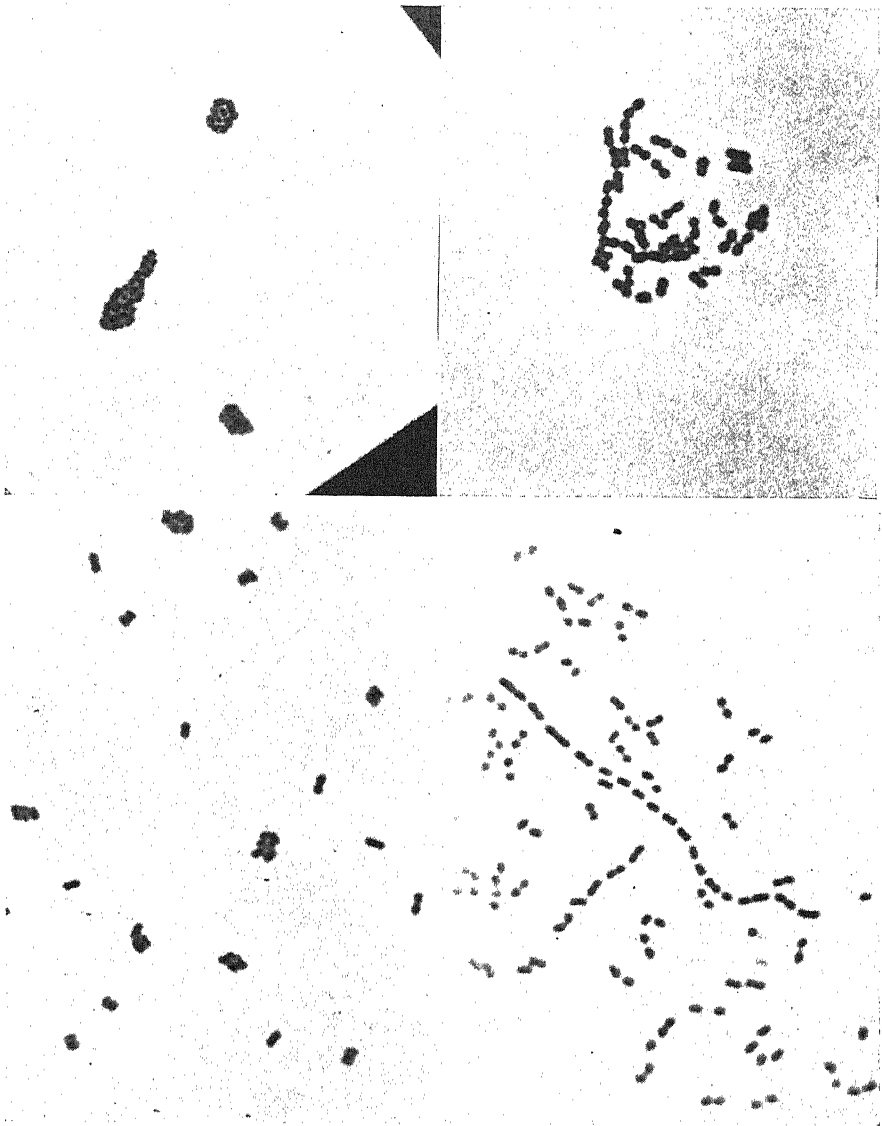


FIG. 3. *Upper left:* Strain 730, A type. *Upper right:* Strain 700, B type. *Lower left:* Strain 200, D type. *Lower right:* Strain 860, F type. From 2% raw sugar broth cultures 1-day old. Stained with carbol fuchsin.  $\times 1,092$ .

tinguish the types. Photomicrographs of representative strains of each group are shown in figure 3. In our experience members of type D are far more abundant in cane juice, fresh or frozen, than are the other types.

Immediately after isolation all strains were tested for ability to ferment sucrose, lactose, xylose, and arabinose. At that time a considerable number of D

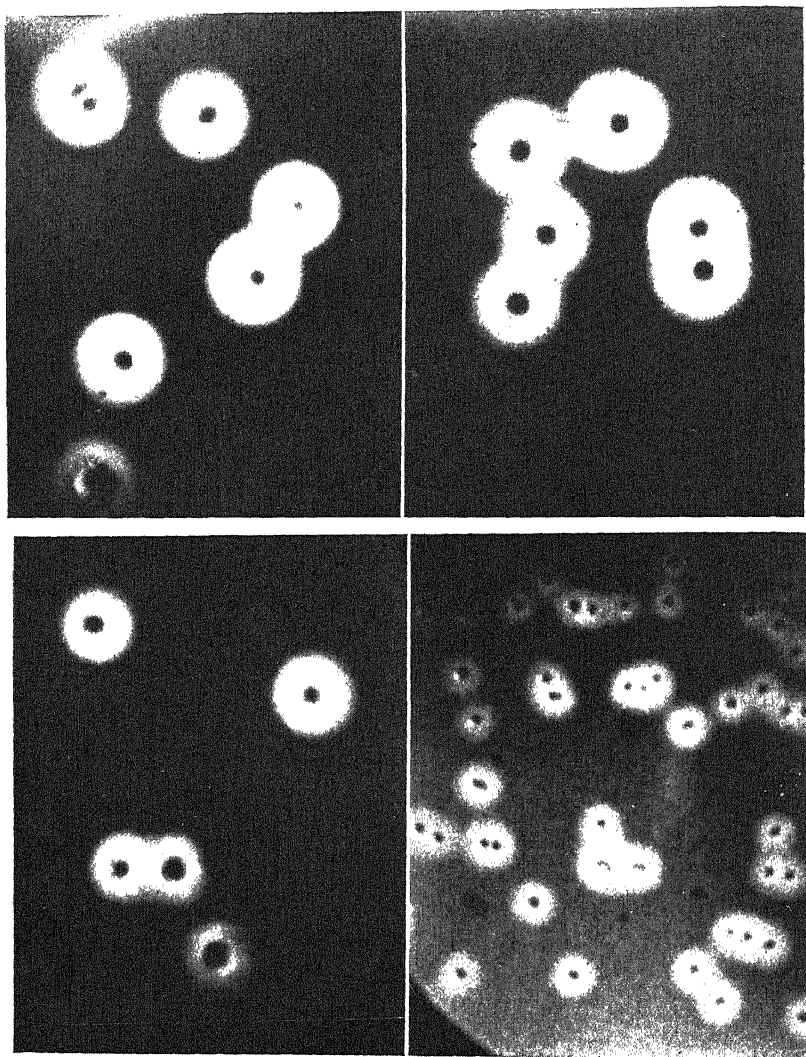


FIG. 4. *Upper left:* Strain 730, A type. *Upper right:* Strain 158, B type. *Lower left:* Strain S35, D type. *Lower right:* Strain 714, F type. Colonies on sheep's blood agar with 0.1% added glucose, incubated at room temperature for 7 days.  $\times 7$ . Only the dense central portion of the colonies shows in the figures. The zone of hemolysis was in most cases less than half the diameter of the colony.

strains failed to ferment sucrose and lactose, but when tested some months later these strains were found to produce a delayed fermentation. Successive trans-

fers to fresh tubes of sugar after acid development reduced the time required for fermentation to 2 to 6 days.

TABLE 2  
*Fermentation reactions*

SUBSTRATE	COLONIAL TYPES AND PERCENTAGE OF STRAINS FERMENTING TEST SUBSTANCES			
	A(18 strains)	B(23 strains)	D(112 strains)	F(15 strains)
Xylose.....	100	100	97*	100
Arabinose.....	100	100	45	100
Glucose.....	100	100	100	100
Fructose.....	100	100	100	100
Galactose.....	100	100	100	100
Mannose.....	100	100	100	100
Sucrose.....	100	100	100*	100
Trehalose.....	100	100	40	33
Maltose.....	100	100	100	100
Lactose.....	100	100	71	100*
Melibiose.....	100	100	46	0
Cellobiose.....	100	100	80	33
Melezitose.....	0	4	32	0
Raffinose.....	100*	100*	40	0
Starch.....	0	4	50*	0
Dextrin.....	0	4	50*	0
Glycogen.....	0	0	0	0
Xylan.....	0	0	0	0
Inulin.....	0	0	0	0
Esculin.....	100	100	94	100
Salicin.....	100	100	72	100
Amygdalin.....	100	96	51	20
Glycerol.....	0	0	0	0
Sorbitol.....	0	0	0	0
Mannitol.....	100*	80*	10*	100*
Dulcitol.....	0	0	0	0
Inositol.....	0	0	0	0
Erythritol.....	0	0	0	0

\* Indicates delayed fermentation by some or all strains.

Of the 740 isolates, 168 were selected for more detailed study. All isolates were gram-positive cocci, and were alike in the following characters:

Catalase negative.

Oxidase negative (*para*-aminodimethylaniline monohydrochloride test).

Methylene blue reduced.

Indole not produced.

Nitrates not reduced.

Voges-Proskauer (Barrett's method) negative (5 days).

No perceptible growth in ordinary nutrient agar or broth.

Acetate, lactate, tartrate, succinate, and citrate not utilized as sole carbon source.

Litmus milk unchanged or long-delayed acid formation; few strains produced coagulation even after 28 days.

Most strains grew in the presence of 4 per cent but not in 6 per cent NaCl.

The fermentation reactions in various sugars, glucosides, and polyhydric alcohols are presented in table 2. The basal medium employed in all fermentations studies consisted of tryptone, 1 per cent; yeast extract, 0.5 per cent; and  $K_2HPO_4$ , 0.1 per cent. The strains of type A were found to constitute a homogeneous group, and B and F were relatively homogeneous, but type D strains varied widely in the ability to ferment these substances. Both xylose and arabinose were attacked by all strains of A, B, and F types, and one or both pentoses were attacked by all strains of type D. Three strains of this group failed to fer-

TABLE 3  
*Characteristics of Leuconostoc mesenteroides*

TYPE	STRAINS TESTED	PERCENTAGE OF STRAINS PRODUCING POSITIVE REACTIONS					
		Viscosity*				Rope†	Fluorescence in 15% sucrose
		1+	2+	3+	4+		
A	18	100	0	0	0	0	0
B	30	27	27	23	0	0	0
D(1)	10	0	0	10	90	0	100
D(2)	30	42	13	3	3	50	0
F	14	0	0	61	39	0	0

4+ = complete solidification; 3+ = consistency of honey; 2+ = consistency of medium syrup; 1+ = slight but perceptible increase in viscosity.

\* Viscosity was determined in 15% commercial sucrose after 8 days' incubation at room temperature (25-28 C).

† Determined in 15% sugar medium after 5 days' incubation at 25-28 C.

TABLE 4  
*The effect of temperature on production of viscosity in 15 per cent sucrose broth*

TYPE	STRAINS TESTED	14 DAYS 8-10 C	7 DAYS 25-28 C	7 DAYS 37 C	7 DAYS 44 C
A	18	2+ to 3+*	1+	0	0
B	30	1+ to 3+	0 to 3+	±	no growth
D	40	1+ to 4+	0 to 4+	±	0
F	16	2+ to 3+	3+ to 4+	+	no growth

\* See footnote to table 3.

ment xylose, and sixty failed to attack arabinose. All the gum-forming isolates were, therefore, identified as *L. mesenteroides* since they fermented sucrose and a pentose.

Wide variations were noted in the ability of strains to produce gum in 15 per cent sucrose media (table 3). When grown at 20 to 30 C members of type F rapidly solidified the 15 per cent sucrose medium, but type D usually required 5 to 10 days to solidify the medium, and some strains never produced solidification. No type A isolate ever produced more than slight viscosity in the 15 per cent sucrose medium, when grown at 20 to 30 C, whereas type B strains produced a variable amount of gum, but never complete solidification.

It was observed that the viscosity developed in 15 per cent sucrose broth was markedly affected by the temperature of incubation (table 4). At 44 C there was no evidence of gum formation by any of the strains. At 37 C type F strains produced a slight increase in viscosity, but other strains produced no appreciable change in viscosity. Gum formation was most active at room temperature with most strains except those of type A, which evidenced greater viscosity when grown at 8 to 10 C. The growth of all strains was much slower at 8 to 10 C, and more time was required for the development of maximum viscosity.

Certain isolates produced gum without developing "rope," but others produced a distinctly ropy condition. Rope was not observed in any of the A, F, or fluorescent D strains, at any age or at any temperature of cultivation. Many of the B strains exhibited ropiness during the first 2 or 3 days of growth at room temperature, and at 8 to 10 C the condition persisted for at least 10 days. At room temperature certain of the nonfluorescent strains of type D produced ropiness which persisted indefinitely. It was also noted that, in the D type, most of the fluorescent strains produced large amounts of gum, whereas the nonfluorescent strains were, as a group, less active in gum formation.

The phenomenon of fluorescence was never observed except in certain strains of type D, and in these strains only when grown in 10 or 15 per cent sucrose, and after a considerable amount of gum had been formed. These cultures appeared yellowish green or bluish green when viewed by reflected light. In certain cultures of types A and B grown in 15 per cent sucrose broth a slightly blue opalescence was noted, particularly at the meniscus. This is probably similar to the opalescence of *Streptococcus salivarius* cultures reported by Niven, Kiziuta, and White (1946). This condition is not to be confused with the phenomenon of fluorescence noted in certain D strains.

The fluorescent strains of type D are possibly identical with the cultures described as *Leuconostoc dextranicum* type I by Alford and McCleskey (1942), since both are strong producers of gum (solidifying 15 per cent raw sugar solutions), are fluorescent, and do not produce evidence of gas in the gum. The cultures and research notes of Alford and McCleskey were lost during the war, and it has, therefore, not been possible to include their strains in this study. Their cultures, reported as negative in xylose, were probably only slow in pentose fermentation (as were some of ours), which fact led them to consider their isolates to be *L. dextranicum* rather than *L. mesenteroides*.

When the isolates were grown at room temperature in high (5 to 15 per cent) concentrations of sucrose to observe gum-forming ability, it was noted that many of the B strains and all of the F strains produced visible evidence of gas. Under these conditions gas was never observed in A and D strains. In Durham fermentation tubes with 10 per cent sucrose, however, the A strains were revealed as active gas producers (table 5). Strains of type D never produced evidence of gas except when cultured under the paraffin seal, and then only a very small amount was formed. Only one isolate failed to produce even a bubble of gas under the seal.

The amount of gas produced varied widely among the strains of each type,

but no member of the D type produced so much as 10 per cent gas, whereas the maximum for the A, B, and F types (under seal) was 50, 70, and 95 per cent, respectively. The F strains were by far the most active gas formers in sucrose media.

The cultivation of the isolates at low pH and at a high temperature revealed further differences among the types (table 6). At pH 4.1 all isolates grew, but at pH 3.75 none of the A strains and only 2 of the D strains initiated growth. At pH 8.5 about half the B strains failed to grow, but practically all isolates of the other types grew. When incubated at 8 to 10 C all strains grew, but most of

TABLE 5  
*Gas production by Leuconostoc mesenteroides*

TYPE	STRAINS	PERCENTAGE OF STRAINS PRODUCING VISIBLE EVIDENCE OF GAS				AVERAGE VOLUME OF GAS (%)*
		Durham fer. tubes		15% sucrose, no seal	5% sucrose, paraffin seal	
		1% sucrose	10% sucrose			
A	18	0	83.4	0	100	24
B	29	3.4	69.0	57	100	23
D	38	0	0	0	97.4	4
F	14	0	100	100	100	37

<sup>\*</sup> Results obtained from tubes with paraffin seal.

TABLE 6  
*Limiting temperature and pH for growth of Leuconostoc mesenteroides*

TYPE	STRAINS TESTED	PERCENTAGE OF STRAINS GROWING AT					
		pH 3.75	pH 4.1	pH 8.5	8-10 C	37 C	44 C
A	18	0	100	94.4	100	100	50
B	30	37.5	100	53.1	100	100	0
D	40	5	100	97.5	30 <sup>*</sup>	100	74
F	16	86.6	100	100	100	100	0

All readings were made after incubation for 3 days.

<sup>\*</sup> All strains were growing after 9 days.

the D isolates developed very slowly and did not show evidence of growth within 3 days. At 44 C (air temperature) B and F strains were inhibited, but many of the A and D strains grew. When tested at 45 C in a water bath, none of the A isolates and only 4 of the D strains grew.

The final acidity developed in 15 per cent sucrose broth was found to be greatest at room temperature (25 to 30 C) in all groups (table 7). This temperature was found to be very favorable for gum production also (table 4), but it is apparent that high acid formation is not always accompanied by the formation of large amounts of gum. Greater viscosity in every instance occurred at 8 to 10 C with relatively low acidity rather than at 37 C with high acidity.

Isolates of type D were found to vary quite widely in final acidities produced in



sucrose broth, whereas the other groups showed much less variation. Although somewhat atypical strains were found in all types, it appears that type D is far more heterogeneous than the other groups.

On sheep's blood agar containing 0.1 per cent added glucose the different groups could not be clearly distinguished. The colonies of types A, B, D, and F were 0.7 to 2.0 mm in diameter, slightly raised, and gray in color. Colonies of the F type were generally somewhat smaller than those of the other types. After 2 to 7 days all A, B, and D strains produced distinct hemolysis, whereas some of the strains of type F produced slight or no hemolysis (figure 4).

## SUMMARY

*Leuconostoc mesenteroides* isolates from cane juice were found to consist chiefly of four relatively distinct colonial types when grown on 10 per cent raw sugar agar at 20 to 28 C. These types were found to differ also in certain fermentation reactions, in amount of gum, gas, and acid produced, and in the temperature and pH requirements for growth.

TABLE 7

*The effect of temperature on the final pH in 15 per cent sucrose broth*

TYPE	STRAINS TESTED	16 DAYS 8-10 C			16 DAYS 25-30 C			10 DAYS 37 C			8 DAYS 44 C		
		Low	High	Avg	Low	High	Avg	Low	High	Avg	Low	High	Avg
A	18	4.05	4.35	4.25	3.61	3.78	3.70	3.70	3.89	3.80	5.25	6.2	5.58
B	28	4.0	4.68	4.27	3.60	3.82	3.68	3.75	4.02	3.89	no growth		
D	38	4.14	5.45	4.77	3.97	4.25	4.12	3.95	4.70	4.15	4.4	5.8	4.87
F	14	4.0	4.30	4.05	3.86	3.95	3.90	3.80	4.05	3.90	no growth		

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# THE APPLICATION OF BACTERIOPHAGE AND SEROLOGY IN THE DIFFERENTIATION OF STRAINS OF *LEUCONOSTOC MESAENTEROIDES*

ALVARO LEIVA-QUIROS AND C. S. McCLESKEY

*Louisiana State University, Baton Rouge, Louisiana*

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The bacterial species *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem as characterized by Bergey *et al.* (1939) includes four strains which are markedly different in colonial appearance when grown on sucrose agar. These strains, or types, have been designated A, B, D, and F by Faville (1947) and McCleskey, Faville, and Barnett (1947). These types also differ significantly in certain fermentative reactions and in the amount of gum produced. The purpose of the work reported in this paper was to determine whether or not these four types of *L. mesenteroides* comprise distinct entities as revealed by serological reactions and lysis by specific bacteriophages.

Some years ago Hucker (1932) attempted to utilize the agglutination reaction in the separation of species of the genera *Leuconostoc* and *Streptococcus* and concluded that these species "evidence a large amount of strain specificity." He found cross reactions between *Leuconostoc mesenteroides* and various streptococci to be common. Neill and associates (1941) have reported a serological relationship between the polysaccharides of *L. mesenteroides* and the type II pneumococcus.

In the serological studies both precipitation and agglutination methods were employed. For the precipitin tests antisera were prepared by injecting heat-killed cells intravenously into rabbits in accordance with the procedure of Lancefield (1933). The centrifuged bacterial sediment from 50 ml of a 24-hour culture grown in tryptone glucose yeast extract broth was resuspended in half volume of saline, and the cells were killed by heating at 56 C for 1 hour. One to 2 ml of this suspension were injected daily for 7 days, followed by a week's rest period. Three series of injections were given. On the fifth day after the last injection the rabbits were bled, and the separated sera were stored in the icebox until used.

The antigen extracts were prepared by a modification of the Lancefield technique. The bacterial sediment from 50 ml of a 24-hour broth culture was suspended in 2 ml of N/20 HCl in 0.85 per cent saline. The tube was immersed in a water bath at 60 C for 1 hour and then centrifuged. The supernatant liquid was removed and to it was added a drop of 0.04 per cent bromthymol blue. It was then neutralized with N/2 NaOH, centrifuged, and the sediment discarded. The remaining supernatant contained the antigen and was used in the precipitin test.

The tests were made in very small tubes, in which 0.05 ml of serum were placed and 0.05 ml of antigen layered over it. The tubes were examined for ring formation after 30 minutes at room temperature and again after 30 minutes at 37 C

in the water bath. The contents of the tubes were then mixed, placed in the refrigerator, and observed the next day. All sera were controlled by testing with the homologous antigen and with a saline control.

In table 1 are presented the results of the precipitin tests using a total of 29 extracts. Of type A two extracts were tested against four sera; two were precipitated by 730(A) serum and none by sera 11(D), 548(D), and 860(F). Five type B extracts were tested of which 2 gave positive reactions with serum 730(A), 1 with serum 11(D), 2 with serum 548(D), and none with serum 860(F). In the D type 16 extracts were used, of which none was precipitated by serum 730, 2 by serum 11(D), 2 by serum 548(D), and none by serum 860(F). In the F type

TABLE 1

*The precipitin test applied to the differentiation of Leuconostoc mesenteroides strains*

TYPE	STRAINS TESTED	PER CENT POSITIVE PRECIPITATION REACTIONS			
		730(A)	11(D)	548(D)	860(F)
A	2	50	0	0	0
B	5	40	20	40	0
D	16	0	12.5	12.5	0
F	6	16.6	16.6	16.6	33.3

TABLE 2

*Agglutination reactions in the Leuconostoc mesenteroides group*

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH ANTISERUM					
		730(A)	158(B)	11(D)	1060(D)	548(D)	860(F)
A	18	100*	0	0	0	0	5.5
B	30	0	6.6	0	0	0	0
D	50	22	10.5†	28	54	34	26
F	17	11.8	23‡	5.9	0	0	94.1

\* Percentages are based on agglutination at 1:100 dilution.

† Thirty-eight strains tested.

‡ Thirteen strains tested.

we used 6 extracts, of which 2 were precipitated by serum 860(F) and 1 by each of the other sera (the same extract in each case).

For the agglutination tests the same sera were used. Four different dilutions were employed—1:50, 1:100, 1:200 or 1:400, and 1:600 or 1:1,000. The highest dilution employed was in each case the titer for the homologous organism. The tests were conducted in 0.5 per cent saline to reduce the likelihood of spontaneous agglutination. The antigens were 24- to 48-hour tryptone glucose yeast extract broth cultures.

Table 2 shows the results obtained in the 1:100 dilution or higher. Although there was cross agglutination between the four types, there was some evidence that certain types constitute reasonably distinct serological groups. Type A

is apparently the most homogeneous, serologically, of the four types. The B strains were not agglutinated by any serum other than anti-B, but our single serum of that type agglutinated only one culture in addition to the homologous organism.

The results obtained in agglutinin absorption tests further confirm the serological homogeneity of the type A strains (table 3). Absorption with the F type

TABLE 3  
*Agglutinin absorption tests with antiserum 730 (type A)*

ANTIGENS		UNABSORBED	ABSORBED WITH 1064(D)	ABSORBED WITH 864(F)
Type A	18 strains.....	400*	400	400
Type D	1,068.....	200	0	100
	1,061.....	100	0	100
	1,063.....	200	0	0
	1,064.....	400	0	100
	1,067.....	200	0	100
	956.....	200	200	200
	1,065.....	400	0	100
Type F	860.....	100	0	0
	861.....	50	0	0
	864.....	50	0	0
	1,010.....	400	100	0

0 Indicates no agglutination.

\* Numbers indicate highest dilution of serum causing agglutination.

TABLE 4  
*Absorption tests with antiserum 158 (type B)*

TYPE	CULTURE NUMBER	158 B NOT ABSORBED	158 B ABSORBED WITH 1064(D)
B	164	400	200
	158	400	400
D	1,061	400	400
	1,064	400	100
	1,067	400	400
	1,068	400	200
	1,010	100	100
F	1,011	100	100
	1,012	100	100

strain failed to remove the agglutinins for the A type but effectively removed the agglutinins for all the F types and partially removed those for the D type as well. Similarly absorption with the D type strain removed none of the A agglutinins but removed the agglutinins for most of the D and F types. Absorption tests with the anti-B serum using a D strain resulted in lowered titer for a B strain and a D strain, with no effect on the homologous B strain and the F strains (table 4). Likewise absorption of the anti-F serum with a D antigen had little effect on the titer for either A, D, or F strains (table 5).

For the isolation of the different bacteriophages, "mud" from the L. S. U. sugar house was diluted in about an equal portion of water and allowed to settle for 2 days. The supernatant fluid was first filtered through paper and then through a Pasteur-Chamberland filter. The filtrate obtained was inoculated into a series of tubes seeded with the different strains of *L. mesenteroides*. Each tube containing tryptone glucose yeast extract broth was seeded with 1 loopful of one of the strains and inoculated with 1 ml of the filtrate. A control tube without the filtrate was inoculated with each organism. The tubes were observed after

TABLE 5  
*Agglutinin absorption tests with antiserum 860 (type F)*

		ANTISERUM 860F	
		Not absorbed	Absorbed with 956(D)
Type A	153 .....	50	50
	732 .....	100	50
	1,021 .....	50	50
	1,022 .....	50	50
	1,024 .....	50	50
Type D	1,067 .....	100	0
	835 .....	200	200
	910 .....	200	200
	956 .....	400	0
	320 .....	200	200
Type F	1,010 .....	400	400
	1,011 .....	200	200
	1,012 .....	100	200
	860 .....	400	400
	861 .....	100	200
	862 .....	400	400
	864 .....	200	200
	866 .....	200	200
	867 .....	200	200
	868 .....	50	50
	708 .....	100	100
	711 .....	100	200
	714 .....	50	200

1 and 2 days for lysis, and those which showed no growth of the organism were reinoculated with a growing, young culture. If no growth occurred, the bacteriophage suspension was filtered through a Pasteur-Chamberland filter and, after several repetitions of this procedure, the filtrate was used for the tests.

Considerable difficulty was experienced in isolating the bacteriophages, and only 5 were obtained. Each bacteriophage was tested for the formation of plaques with the homologous organism and then tested against all the strains available. The bacteriophages obtained were for the strains 730 (A type), 700 and 706 (B type), and 200 and 209 (D type). No bacteriophage was obtained for any organism of the F type.

The lytic activity of the bacteriophages was determined by the spot plate

method. Petri plates were poured with glucose tryptone yeast extract agar (1 per cent agar) and after solidification were allowed to dry for 4 hours in the incubator at 37 C. Then 2 drops of a young broth culture were spread on the surface by means of a glass rod, and the plates were again allowed to dry for 4 hours. Inoculation with phage was effected by depositing a loopful of active filtrate on a certain spot on the plate. All the bacteriophages were tested on the same plate.

The results of this experiment are presented in table 6. It is apparent that each phage tested exhibited type specificity to a considerable degree. Of 105 strains only two were lysed by phages from outside the type. A high degree of strain specificity was noted, however, particularly in the D type.

TABLE 6  
*Susceptibility of Leuconostoc mesenteroides to bacteriophages*

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH PHAGE				
		730(A)	700(B)	706(B)	200(D)	209(D)
A	18	50	0	0	5.5	0
B	31	3.2	41.9	41.9	0	0
D	42	0	0	0	11.9	9.5
F	14	0	0	0	0	0

#### SUMMARY

Serological and bacteriophagic studies on strains of *Leuconostoc mesenteroides* isolated from cane juice indicated that Faville's type A constitutes a reasonably distinct and homogeneous group, whereas the B, D, and F types are quite heterogeneous. Of the three tests employed the agglutination test was the most useful in showing type relationships.

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# REDUCING THE PYROGENICITY OF CONCENTRATED PROTEIN SOLUTIONS

WILLIAM ELLIOTT SMITH AND ROBERT B. PENNELL<sup>1</sup>

*Department of Immunochemistry, Medical Research Division, Sharp and Dohme, Inc.,  
Glenolden, Pennsylvania*

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Heretofore the removal of bacterial pyrogens from concentrated protein solutions has not been a practical procedure. Zittle and coworkers (1945) in a study of pyrogen removal from protein hydrolyzates found that although Republic S-3 pads effectively removed pyrogens when the solute was of small molecular weight (acid hydrolyzates), these pads were effective only with tremendously expanded filter pad surface area when the solute was of high molecular weight (enzymic hydrolyzates). Similarly, Republic S-3 pads or any of the Seitz type filter pads described as useful for pyrogen removal from crystalloid solutions (Co Tui and Wright, 1942) or from antibiotics (Tonnesen and Vesterdal, 1946) have proved ineffectual in our hands for the removal of pyrogens from protein solutions. The use of charcoal, which has been described as a pyrogen adsorbent (Howard and Spooner, 1946), presents great problems because of the extreme difficulty of its removal from protein solutions.

However, using a new type of Seitz pad, Republic Filter's K-6 and S-6 pads, developed for plasma filtration during the war (Rane and Baldwin, 1945), and an ionic exchange agent, "decalso,"<sup>2</sup> we have been successful in reducing the pyrogenicity of a variety of protein solutions in a manner which permits practical application.

Pyrogen removal, by the technique to be described, has been accomplished with serum, plasma, and hemoglobin solutions ranging from 6 to 12 per cent protein and with normal human serum albumin solution containing 25 per cent protein. Several solutions of each type have been treated successfully. Pyrogen removal is a function of the degree of pyrogenicity and the concentration of the protein solution, and the proper relationship of the amount of "decalso" and filter pad area to these variables. This latter relationship must be evaluated for each solution to be treated, often by preliminary trials. Thus the pyrogenicity of some protein solutions may be reduced by treatment with "decalso" alone, others by filtration alone, whereas some solutions require treatment with increased quantities of "decalso" plus filtration. The data have been selected to illustrate these points.

## PROCEDURE

The pyrogen tests described were all performed by the procedures prescribed by the National Institute of Health for pyrogen tests of biological products

<sup>1</sup> With the technical assistance of Lois Priester.

<sup>2</sup> Manufactured by the "Permutit Co.," New York, New York. Our attention was first drawn to the possible usefulness of "decalso" for pyrogen removal by Dr. R. A. Phillips, Hospital of the Rockefeller Institute.

(Minimum Requirements for Pyrogen Tests on Biologic Products from Blood Serum, Nov. 19, 1945, National Institute of Health).

The pyrogen-free water used in these experiments was prepared by double distillation through Barnstead stills. The water was checked repeatedly for pyrogenicity. All equipment coming in contact with the solutions was carefully cleaned and rinsed with pyrogen-free water before use.

"Decalso" itself occasionally contains a pyrogenic substance. This can be removed by stirring the "decalso" with ten times its weight of a 0.5 per cent solution of sodium carbonate in pyrogen-free water, decanting the supernatant, and washing the "decalso" with pyrogen-free water until the washings are neutral to litmus. All "decalso" used in these experiments was so treated.

In experiments in which the effects of "decalso" alone were to be examined the relatively ineffective S-3 pads were used for sterile filtration.

#### REMOVAL OF PYROGEN FROM HEMOGLOBIN SOLUTION WITH "DECALSO"

A sterile, pyrogenic solution containing approximately 7 per cent hemoglobin gave the following rises above the starting temperatures in the routine pyrogen test.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	1.4 C	1.5 C	-0.3 C	1.5 C
2	1.0 C	0.8 C	0.2 C	1.0 C
3	0.5 C	1.1 C	0.3 C	1.1 C

Two hundred ml of this solution were shaken with 6 g of "decalso" and allowed to stand overnight in the cold (2 C). The solution was then filtered through an S-3 pad, using 100 sq inches of pad per liter of solution. The pyrogenicity of the solution was greatly reduced as shown in the following test.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	0.1 C	0.3 C	0.0 C	0.3 C
2	0.3 C	0.2 C	-0.2 C	0.3 C
3	0.1 C	0.0 C	0.1 C	0.1 C

Five liters of this hemoglobin solution were shaken with 30 g of "decalso" per liter. After standing 4 hours at 2 C, the solution was filtered through an S-3 pad, using a greatly reduced pad surface area, i.e., about 8 sq inches per liter of solution, thereby eliminating for all practical purposes the effect of filtration. It will be seen that the "decalso" itself had an appreciable effect.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	0.9 C	0.9 C	0.9 C	0.9 C
2	0.2 C	0.5 C	0.4 C	0.5 C
3	0.0 C	0.5 C	0.5 C	0.5 C

#### REMOVAL OF PYROGEN FROM PLASMA WITH S-6 FILTRATION AND WITH "DECALSO"

A sterile plasma pool was found to be pyrogenic as shown by the following test.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	0.8 C	0.8 C	0.7 C	0.8 C
2	0.2 C	0.6 C	0.9 C	0.9 C
3	-0.6 C	-1.9 C	-3.2 C	—
4	0.7 C	1.5 C	1.7 C	1.7 C
5	0.5 C	1.3 C	1.4 C	1.4 C

This plasma pool affords an example of a protein solution from which the removal of pyrogenicity was effected by either of the agents alone.

Five hundred ml of this plasma were filtered through an S-6 pad, 40 sq inches per liter. After filtration a pyrogen test showed the following.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	-0.6 C	-0.7 C	-0.5 C	—
2	-0.2 C	0.4 C	0.4 C	0.4 C
3	-0.2 C	0.4 C	0.4 C	0.4 C

Five hundred ml of the pyrogenic plasma were stirred with 15 g of "decalso" for a half-hour and were filtered through an S-3 pad, 40 sq inches per liter. The filtered material gave the following pyrogen test.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	0.2 C	0.4 C	0.5 C	0.5 C
2	-0.3 C	-0.1 C	0.1 C	0.1 C
3	-0.3 C	0.0 C	-0.1 C	—

#### REMOVAL OF PYROGEN FROM A 25 PER CENT NORMAL HUMAN SERUM ALBUMIN SOLUTION

A 25 per cent solution of albumin gave the pyrogen tests below. Its pyrogenicity is better indicated by the fact that one of the rabbits died immediately after the test, and the other two were moribund, than by the actual temperature rises shown.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	0.4 C	0.2 C	0.2 C	0.4 C
2	0.4 C	0.1 C	0.1 C	0.4 C*
3	1.2 C	1.5 C	1.9 C	1.9 C

\* Rabbit died.

Earlier work has shown that with the highly concentrated albumin solutions the 30 g of "decalso" per liter usually used were ineffective even when followed by S-6 filtration. Likewise, the use of 60 g of "decalso" per liter of solution was only slightly effective. Five hundred ml of the foregoing solution were stirred one half-hour with 120 g of "decalso" (240 g per liter), and then were filtered through an S-6 pad, 40 sq inches per liter. The filtered albumin gave the following pyrogen test. None of the rabbits were ill.

<i>Rabbit</i>	<i>1 hr</i>	<i>2 hr</i>	<i>3 hr</i>	<i>Max rise</i>
1	-0.7 C	-0.5 C	-0.3 C	—
2	-0.2 C	-0.4 C	-0.4 C	—
3	-0.8 C	-0.4 C	-0.3 C	—

## SUMMARY

The pyrogenicity of concentrated protein solutions can often be reduced by treatment with "decalso," and by filtration through S-6 pads of the Republic series.

The amount of "decalso" and the area of pad space used are functions of the degree of pyrogenicity and of the protein concentration of the solution, and for maximum effectiveness they should be determined for each solution to be examined. In practice, for 6 to 8 per cent protein solutions of mild pyrogenicity, 30 g of "decalso" and 40 sq inches of pad surface per liter of solution have given adequate reduction of pyrogenicity.

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# CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

## IV. COMPARATIVE RESPONSES OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA TO PENICILLIN<sup>1</sup>

ROBERTSON PRATT AND JEAN DUFRENOY<sup>2</sup>

*University of California College of Pharmacy, The Medical Center, San Francisco 22, California*

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It is well established that in general large differences exist in the sensitivity of gram-positive and of gram-negative organisms to penicillin; the differences are so large, in fact, that most infections due to gram-negative organisms are classed as refractory to penicillin treatment, although it is known that at sufficiently high concentrations of the antibiotic *in vitro* many such organisms are inhibited. It is also well established that there are relatively wide differences in sensitivity to penicillin among different species of susceptible bacteria and even among different strains or races of a single susceptible species (Herrell, 1945). It seems of interest, therefore, to ascertain whether the same mechanism of penicillin action operates in gram-negative organisms as in gram-positive, but perhaps at a higher threshold level, or whether an entirely different mechanism of action must be sought. This problem is of practical as well as theoretical importance, since, if the same mechanism is operative in both types of organisms, but merely at different threshold levels, it may be possible to find a practical means of lowering the threshold of sensitivity in the more resistant organisms and thus to bring them within the scope of effective action of penicillin in practical clinical doses. The advantages to be anticipated from such a procedure are obvious, since the superiority of penicillin over other currently available antibiotics on the basis of toxicity, untoward reactions, development of fastness in the organisms under treatment, etc., is generally recognized.

It is difficult, if not impossible, in the present state of knowledge to define with certainty the precise biochemical and biophysical mechanisms through which penicillin exerts its effect on susceptible organisms. The fact that it exerts a strong selective action in relatively low concentrations against many types of bacterial cells without manifesting any appreciable toxicity toward other living structures indicates that it does not owe its action to a drastic, general protoplasmic poisoning as do many other antibacterials (halogens, salts of heavy metals, phenols, etc.), but it is reminiscent rather of the "receptor" hypothesis of Ehrlich (1908, 1914), who believed that antiseptic agents act against susceptible cells by linking chemically with reactive groups contained therein. Specific tests with different stains and reagents may be visualized, in a way, inasmuch as they help to reveal chemical changes that occur in cells and colonies under the

<sup>1</sup> The execution of the work reported in this paper was made possible by a generous grant from the Cutter Laboratories, Berkeley, California.

<sup>2</sup> With the laboratory assistance of Virginia Lamb.

influence of penicillin, as tending to define the "receptor" systems chemically. No one of the tests alone can be taken as conclusive evidence of the chemical groups or systems involved in inactivation of bacterial cells by penicillin, but the results of a number of such tests with different reagents may be interpreted as convergent lines of evidence that tend to suggest mechanisms of action. The results of such investigations on *Staphylococcus aureus* have provided evidence that a threshold effect obtains on penicillin assay plates and that that effect depends, in part at least, upon an oxidation-reduction threshold. The sharp boundaries at the margins of inhibition zones may be taken as representing visible expressions of an irreversible shift of -SH to S-S in the cytoplasm of the cells (Dufrenoy and Pratt, 1947a). The outlines of the zones of inhibition, where little or no growth occurs, are accentuated by the fact that the bounding rims of the zones are composed of narrow bands in which the growth of the test organisms is enhanced over that of the cells further removed from the central cylinder containing penicillin. These bands occupy a region of the plate within the range of diffusion of appreciable concentrations of penicillin but slightly beyond the range of diffusion of bacteriostatic concentrations. This observation is consistent with the observation that at sufficiently low concentrations *in vitro* penicillin exerts a stimulating effect on microorganisms (Miller, Green, and Kitchen, 1945; Eriksen, 1946; Curran and Evans, 1947).

Techniques similar to those previously employed in this laboratory were used in the present work to study the reactions on penicillin assay plates seeded with the gram-negative organisms *Escherichia coli* and *Proteus vulgaris*, as well as on plates seeded with the gram-positive *Staphylococcus aureus* and *Bacillus subtilis*.

#### MATERIALS AND METHODS

Assay plates were prepared and incubated, except where noted to the contrary under the individual experiments, according to the procedure prescribed for the modified cylinder plate method involving physical development (Goyan, Dufrenoy, Strait, and Pratt, 1947). After being seeded and subjected to suitable periods of preincubation (without penicillin) and of secondary incubation (with penicillin), they were treated according to the techniques previously described (Dufrenoy and Pratt, 1947a,b; Pratt and Dufrenoy, 1947a,b) with appropriate reagents intended to reveal specific reactive compounds or groups, or classes of reactive compounds or groups. Critical examination and study of different parts of plates so treated reveal much information concerning chemical changes that occur in bacterial colonies exposed to lytic, bactericidal, bacteriostatic, and subbacteriostatic concentrations of different agents.

A crystalline preparation of sodium penicillin G that assayed 1,549 units per milligram was used for the experiments. The organisms that were studied were *Staphylococcus aureus* NRRL-313 (same as FDA strain 209P), *Bacillus subtilis* NRRL-B-558, *Escherichia coli*, and *Proteus vulgaris* HX19. It was necessary to use different schedules of incubation for each organism because of their different inherent rates of growth. The purpose of the preliminary incubation is to permit the organisms to reach the logarithmic phase of their growth curve in which

they are most sensitive to penicillin before coming in contact with the antibiotic. In the 3-hour cylinder plate assay for penicillin (Goyan, Dufrenoy, Strait, and Pratt, 1947) it is desirable to choose the length of the second period of incubation, the period during which penicillin is permitted to diffuse on the plates, so as to provide a balance between growth of the organisms (an exponential function of time) and diffusion of antibiotic (a linear function of time) such that the curve relating log diameter of zone of inhibition to log concentration of antibiotic is linear. If too short a period of diffusion is permitted, obviously no zone of inhibition develops. If too long a period of secondary incubation is provided, the biological factor (growth) overrides the physical factor (diffusion) and a nonlinear calibration curve results. Proper control of the secondary period of incubation was important in the present work also, because, if too long a period of contact with inhibiting concentrations of the antibiotic was permitted, extensive lysis occurred in the inhibition zones, and colonies in intermediate stages of inactivation, such as are desirable for cytochemical studies, became very scarce. The most useful schedule of incubation was found to be as follows:

ORGANISM	DURATION OF INCUBATION AT 37 C	
	Primary period (without antibiotic)	Secondary period (with antibiotic)
<i>S. aureus</i> .....	3 hours	3 hours
<i>B. subtilis</i> .....	2 hours	2.5 hours
<i>E. coli</i> .....	3 hours	5 hours
<i>P. vulgaris</i> .....	3 hours	3 to 6 hours

#### EXPERIMENTS AND RESULTS

Results observed following tests with various dyes and reagents on penicillin assay plates seeded with gram-positive and with gram-negative organisms are summarized in tables 1 and 2, respectively.

The experiments have shown that, in general, essentially the same pattern appears on penicillin assay plates subjected to the various stains and reagents after incubation whether they are seeded with the penicillin-resistant, gram-negative organisms *Escherichia coli* and *Proteus vulgaris* or with the sensitive gram-positive organisms *Staphylococcus aureus* and *Bacillus subtilis*. This suggests that penicillin elicits essentially the same responses in the two classes of organisms if appropriate concentrations are employed (see below). It is interesting to note in this connection that, using a different technique, Schuler (1947) concluded that penicillin acts on gram-positive and on gram-negative organisms through the same mechanism.

Each zone of inhibition on the assay plates is bounded by a clearly discernible narrow ring formed by a very abundant growth of the test organisms that markedly exceeds the growth on other portions of the plate. Since a diffusion gradient must exist around the cylinders containing the penicillin solutions, these regions of enhanced growth immediately outside the zones of inhibition may be interpreted as representing areas in which the concentration of penicillin, although

TABLE 1  
*Response to different dyes and reagents on penicillin assay plates seeded with gram-positive organisms*

REAGENT	GROUP ASSUMED TO BE ACTIVE	AUTHORITY* AND DESCRIPTION OF TEST	REACTION ON ASSAY PLATES					
			<i>Staphylococcus aureus</i>			<i>Bacillus subtilis</i>		
			Color		Definition of boundary	Color		Definition of boundary
			Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K-ferricyanide, ferric sulfate	-SH	Mason, H. L., 1930	Faintly bluish	Deep blue	Very sharp	Faintly bluish	Deep blue	Very sharp
Schiff's	Free aldehydes	Oster, K. A., 1946	Clear, faintly pink	Red	Very sharp deep red ring of enhanced growth	Clear, faintly pink	Deep red	Sharp
Schiff's (after pretreatment of plate with HgCl <sub>2</sub> )	Aldehydes bound in cells	Oster, K. A., 1946	Faintly pink	Deep red	Very sharp	Test not performed		
Osmic acid	Dienol (o-polyphenols)	Dufrenoy, J., 1945	Clear	Dark	Very black ring	Clear	Dark	Sharp black ring
Azo-reaction (in alkaline solution)	Dienol (o-polyphenols)	Lison, L., 1933	Faintly pink	Red	Very sharp	Orange	Red	Very sharp, brilliant orange-red ring of enhanced growth
Sakaguchi†	Substituted guanido	Sakaguchi, S., 1925; Vincent, D., & Brygoo, P., 1946	Clear	Pink	Very sharp	Faintly pink	Pink	Poor
Molybdate	PO <sub>4</sub> <sup>=</sup>	MacDougal, D. T., & Dufrenoy, J., 1944	Clear gray-blue	Blue	Very sharp deep blue ring of enhanced growth	Golden	Blue	Sharp by transmitted light, poor by reflected light
Hematoxylin after molybdate	Lipidic complex	MacDougal, D. T., & Dufrenoy, J., 1944	Light blue	Deep purple	Moderately sharp. Black	Faint violet	Deep violet	Poor
Toluidine blue	Ribonucleic acid	Jeener, R., & Brachet, J., 1943	Lavender	Purple	Very sharp	Faintly bluish	Deep blue	Sharp
FeCl <sub>3</sub>	o-Diphenols	Lemoigne, M., 1923	Light gray-green	Greenish	Poor	Faintly bluish	Dark blue-green	Sharp
Methyl green (approximately 0.02% solution)	Polynucleotides dehydrogenase systems	Brachet, G., 1942	Green	Pink	Sharp	Faintly greenish	Pink	Poor

\* For complete citation see list of references at the end of the paper.

† Plates must be fixed before reagents are applied.



TABLE 2

*Response to different dyes and reagents on penicillin assay plates seeded with gram-negative organisms*

REAGENT*	REACTION ON ASSAY PLATES					
	<i>Escherichia coli</i>			<i>Proteus vulgaris</i>		
	Color		Definition of boundary	Color		Definition of boundary
	Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K-ferricyanide, ferric sulfate	Faintly blue-green	Deep blue-green	Very sharp, deep blue ring of enhanced growth	Bluish	Blue	Poor
Schiff's	Faintly pink	Pink	Poor	Pink	Deep red	Very sharp deep rose ring of enhanced growth
	Pink†	Red†	Sharp†			
Schiff's (after pre-treatment with HgCl <sub>2</sub> )	Pink	Deep red	Sharp	Pink	Deep red	Extremely sharp
Osmic acid	Clear	Dark	Very sharp black ring	Clear	Dark	Very sharp black ring of enhanced growth
Azo-reaction (in alkaline solution)	Clear	Red	Sharp	Light orange	Red-orange	Sharp
Sakaguchi‡	Faintly pinkish	Pink	Poor	Faintly pinkish	Red-orange	Poor
Molybdate	Blue	Deep blue	Poor§	Blue	Light blue	Good
Hematoxylin after molybdate	Blue-violet	Deep purple	Good	Bright blue-violet	Deep blue (very little violet)	Sharp
Toluidine blue	Lavender	Purple	Sharp	Bluish lavender	Dark purple	Fair
FeCl <sub>3</sub>	Light yellow-green	Greenish brown	Extremely sharp	Light yellow-green	Greenish-brown	Good
Methyl green (approximately 0.02% solution)	Green	Pink	Sharp; intense deep pink ring of enhanced growth	Green	Pink	Very sharp, intense ring of enhanced growth

\* See table 1 for groups assumed to react and for authorities.

† Reaction on plates incubated 18 hours with no preincubation.

‡ Plates must be iced before reagents are applied.

§ Although definition of zones is poor when plates are viewed macroscopically, especially by reflected light, they are very clearly seen when plates are examined under high dry power of microscope. The cells within the zones of inhibition appear as long blue filaments and are sharply differentiated from the uninhibited cells in the normal background. Both filamentous and normal cells stand out clearly from the less intensely stained agar background.

remaining subbacteriostatic, does reach a critical level that is capable of stimulating metabolism and growth. It is well known that, like other antibacterial agents, penicillin in certain subbacteriostatic concentrations may exert a stimu-

lating effect on microorganisms *in vitro* (Miller, Green, and Kitchen, 1945; Eriksen, 1946; Curran and Evans, 1947).<sup>3</sup> The threshold concentration below which penicillin enhances and above which it inhibits metabolism is, however, many times greater for activity toward the gram-negative organisms than toward the gram-positive. For example, on plates seeded with *S. aureus* and treated as prescribed for the FDA cylinder plate assay (Federal Register, 10, 11478-11485,

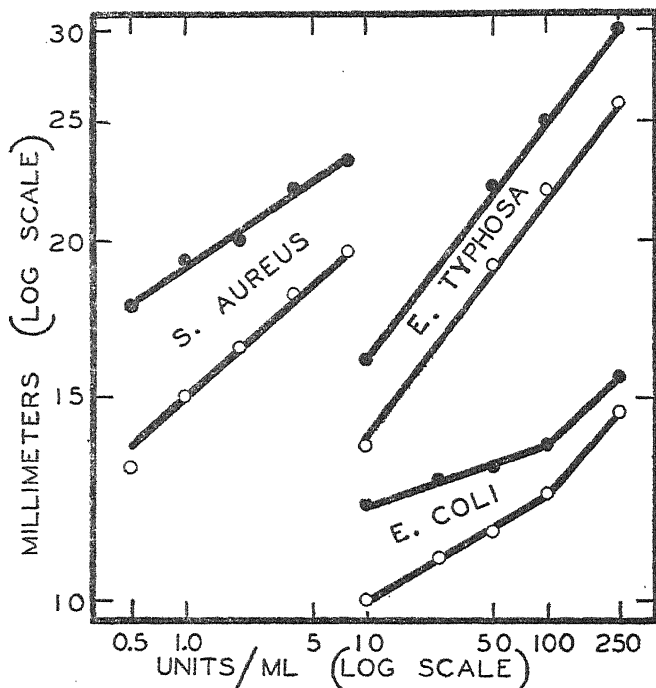


FIG. 1. DIAMETERS OF ZONES OF INHIBITION CORRESPONDING TO DIFFERENT CONCENTRATIONS OF PENICILLIN ON ASSAY PLATES SEEDING WITH *S. aureus*, *E. typhosa*, or *E. coli*

Open circles are values for plates prepared with the standard test agar. Solid circles are for same agar with  $\text{COCl}_2 \cdot 6\text{H}_2\text{O}$  added at the rate of 1 mg/L. All points are averages of values from at least 4 plates. Assays with *S. aureus* were performed by the 3-hour method, others by standard overnight procedure. For convenience in plotting, all values for *E. coli* have been raised 1 mm on the ordinate scale.

1945), a solution containing 1 unit of penicillin per milliliter produced inhibition zones approximately 21 mm in diameter; but a solution containing approximately 100 units of penicillin per milliliter was required to produce a zone of the same diameter on plates seeded with *E. typhosa* or *P. vulgaris*, while solutions containing 250 units per milliliter produced zones only slightly over 13 mm in diameter on plates seeded with *E. coli* (figure 1). It is noteworthy that the addition of

<sup>3</sup> Evidence of similar action of penicillin *in vivo* is less convincing. The authors know of no clear-cut demonstration of such an effect in animals or human patients infected with penicillin-susceptible organisms and treated with penicillin.

trace amounts of cobalt to the test agar markedly lowers the threshold concentration (Dufrenoy, Strait, and Pratt, 1947). Figure 1 shows that the response-dosage curve is shifted along the abscissa when *E. coli* or *E. typhosa* is used as the test organism instead of *S. aureus* and that the curve for a given organism is shifted upward on the ordinate scale when appropriate concentrations of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  are added to the test agar. A similar effect of cobalt in lowering the effective threshold for penicillin has been demonstrated *in vivo* (Pratt, Dufrenoy, and Strait, 1948). The enhancing action of trace amounts of cobalt on penicillin activity appears to be specific and is receiving special study in this laboratory. Salts of nickel, manganese, platinum, iridium, gold, zinc, and copper have been tested in a similar manner over a wide range of concentrations and have failed to produce any similar increase in the diameters of the inhibition zones around cylinders containing penicillin.

#### DISCUSSION

The tests that are most useful in revealing the chemical changes that occur on penicillin assay plates are those that result in clear-cut differential staining of test organisms vs. agar background and of inhibition zones vs. the general background of uninhibited growth, and that intensify the ring of enhanced growth. These staining reactions, emphasizing the contrasts between the regions of normal growth in the background of the plates, the marginal rings of enhanced growth, and the zones of inhibition, can be interpreted from the physicochemical point of view as manifestations of differential shifts in rH and concomitant shifts in pH in the corresponding parts of the plates. The various levels of rH and of pH can be estimated by the proper use of indicators. From the biochemical point of view these changes may probably be ascribed in large measure to changes in the relative rates of proteogenesis and proteolysis in cells exposed to bactericidal, inhibiting, stimulating, and ineffective concentrations of penicillin. Disturbance of the normal assimilative and growth metabolism might be expected to lead, in turn, to an unequal distribution of different proteins and other cellular components on different parts of the plates. As shown in tables 1 and 2 these changes can be revealed by use of reagents for detecting -SH groups, aldehydes, polyphenols, guanido groups, phosphate ion, polynucleotides, fatty acids and lipids, etc. Surface phenomena, due no doubt in part at least to changes in pH, cannot be ignored in interpreting these results and warrant a full discussion separately.

In our experiments it was convenient to work with inhibition zones approximately 15 to 20 mm in diameter. It was found in our work that to produce inhibition zones in this range on plates that were seeded with *E. typhosa*, or *P. vulgaris*, and to which no cobalt had been added, it was necessary to employ penicillin solutions 10 to 100 times as concentrated as when *S. aureus* was used for the test organism, and that, on plates seeded with *E. coli*, solutions containing as much as 250 units of penicillin per milliliter failed to produce zones of this size. However, when the concentration of penicillin was adjusted so that it fell within a bacteriostatic range, the reactions for the several active groups, levels of rH and pH, etc., were as pronounced and sharp on plates seeded with gram-negative

organisms as on those seeded with gram-positive organisms, and they demonstrated a homologous pattern. In fact, plates treated with a given reagent appeared macroscopically identical, irrespective of the test organism that was used, if the relative times of preincubation and of secondary incubation were chosen so that the plates were developed at the time when the sharpest differential could be achieved.

For clearest results it is essential that the interaction of the biological and the physical factors be properly balanced. Our experience indicates that failure of a reagent that has revealed a sharp definition of zones on plates seeded with a given organism to "develop" properly plates seeded with another organism may be ascribed primarily to too long a preincubation period. If the primary incubation period exceeds the duration of the lag period, growth of the test organisms on the plates becomes too dense before the penicylinders<sup>4</sup> are placed thereon and diffusion of penicillin is permitted to begin. Under these conditions the sub-bacteriostatic effect corresponding to "below threshold" concentrations of penicillin may fail to be differentiated from the bacteriostatic effect corresponding to "above threshold" concentrations. For example, the Sakaguchi test (tables 1 and 2) very clearly revealed the prevalence of substituted guanido groups in the zone of enhanced growth on penicillin assay plates seeded with *S. aureus*. The failure of the reagent to provide a sharp response on plates seeded with other test organisms may be ascribed to improper timing of preincubation or secondary incubation periods.

Methods that require flooding of the test plates with reagent solutions are subject to the criticism that the flooding operation may dislodge some of the test organisms from their initial position on the plates and that, consequently, the pattern which develops following the chemical treatment may fail to correspond to the original pattern of distribution of the several reactive groups. Therefore, to eliminate this objection, in the present experiments all results obtained with the several reagents were checked as to sizes of inhibition zones, distribution of enhanced growth, bacteriostasis, and bacteriolysis, on plates which, at the end of the second incubation period, were inverted over a watch glass containing a solution of osmic acid that was stabilized by chromic acid. It was seen that the results on plates so treated corresponded with the observations made on plates treated with the other reagents. The development of the plates exposed to the vapors of osmic acid can be watched easily as it progresses—first, the ring of enhanced growth darkens, and then it blackens as the general background darkens. Microscopical examination of such plates under oil immersion shows that osmic acid is reduced in the vacuolar solution of the test organisms. The reduction occurs more rapidly and is more evident in the zones of enhanced growth. Plates thus "fixed" by fumes of osmic acid may subsequently be stained by appropriate cytological stains, for further study of the cytochemical structure. Osmic acid is reduced rapidly to black osmium oxide in the vacuolar solution and

<sup>4</sup> Trade name for standard cylinders used in assaying penicillin solutions by the agar plate method. Penicylinders are available from Eimer and Amend, New York, and from other firms that supply laboratory apparatus.

in other parts of living cells that contain phenolic materials. To a certain extent it may also be reduced in contact with certain fatty materials. The formation of the black deposit in contact with phenolic compounds has never been clearly explained in terms of physical chemistry. In view of what happens with other metals chelation might be surmised.

The results of our experiments suggest that in penicillin-sensitive organisms the gram-positive complex, which is known to consist of a magnesium ribonucleate involving a sulfhydryl group (Henry and Stacey, 1946; Bartholomew and Umbreit, 1944), accelerates the action of the penicillin molecules in inactivating -SH groups which form essential links in the chain of metabolic reactions involved in growth. Under the effect of penicillin the "gram-positiveness" disappears. This is significant in view of the hypothesis that has been developed in this and in earlier papers (Dufrenoy and Pratt, 1947a; Pratt and Dufrenoy, 1947b), since it is known that the gram-positive complex loses its characteristics as its -SH groups become dehydrogenated to S-S (Henry and Stacey, 1946). In microorganisms which lack the gram-positive complex the concentration of penicillin must be increased many times to obtain the bacteriostatic effect; but irrespective of the minimum dosage required to produce bacteriostasis, the sequence of events is always the same: first the microorganisms undergo a phase of enhanced activity, during which they develop pronounced reducing power, and the cells at the margins beyond the range of diffusion of bacteriostatic concentrations of penicillin manifest the characteristic symptoms of the logarithmic phase of growth. This is the period during which the dehydrogenases are most active, and the rH of the medium tends to drop to the lowest value. This change is evidenced by reduction of Redox indicators. During this phase of growth the organisms store phenolic compounds in their vacuolar solutions which, therefore, acquire the aptitude to absorb (or adsorb) basic fuchsin, phenosafranine, neutral red, etc. The phenolic compounds can be demonstrated by the action of mild oxidants such as potassium dichromate or potassium iodate, which oxidize them to brownish yellow quinoid derivatives. Conversely they can also be demonstrated in the vacuolar solution of the bacteria by virtue of their reducing action toward osmic acid, silver nitrate, etc., or by the formation of darkly colored metallic complexes with ammonium molybdate (Marchal and Girard, 1947) or with ferrous salts. Where positive reactions can be obtained for phenolic compounds, a sharp positive reaction can be obtained for -SH groups through the formation of Prussian blue, by treatment with potassium ferricyanide followed by ferric sulfate.

One other point should be mentioned in connection with the observation that a ring of enhanced growth always surrounds the zones of inhibition on penicillin assay plates. It has been pointed out above that these rings of enhanced growth probably represent a visible manifestation of enhanced metabolism induced in cells in that region by subbacteriostatic concentrations of penicillin. It should be observed, however, that other factors may contribute to the enhancement of growth in those areas circumjacent to the areas where growth is inhibited and lysis of cells occurs. It is not impossible that as cells in the areas of inhibition

are affected by bacteriostatic concentrations of penicillin, some of their components are liberated into the agar, through which they may diffuse to regions of the plate in which the concentration of penicillin fails to reach a bacteriostatic level. A number of references in the literature indicate that products liberated by dying microorganisms may serve as growth factors for survivors (Nicolle and Faguet, 1947; Lasfargues and Delaunay, 1947; Cook and Cronin, 1941; Loofbourow, 1947; Webb and Loofbourow, 1947). Some of these substances, especially the nucleoproteins, might then be absorbed by the bacterial cells outside of the zones of inhibition and serve as metabolites or growth factors. Support for such a hypothesis is afforded by the experiments of Bonét-Maury and Perault (1945). By the use of a recording photometer they observed that when *S. aureus* was cultured in the presence of small amounts of penicillin in broth two waves of growth occurred. The results may be interpreted as indicating that when *S. aureus* cells are suspended in broth containing very small amounts of penicillin, the most sensitive organisms, which are first affected, release into the medium substances that promote a second wave of growth among the more resistant cells. Therefore, the possibility should not be overlooked that the enhancing action that is apparent as a "space" effect on assay plates is comparable in some measure to the action which the recording photometer demonstrates as a "time" effect in suitable broth cultures.

#### SUMMARY

A study has been made of physical and chemical changes that occur in different parts of penicillin assay plates seeded with gram-positive and with gram-negative test organisms. The techniques that were used were intended to reveal differential changes that occur in cells under the influence of bactericidal, lytic, inhibiting, and stimulating concentrations of penicillin as contrasted with the reaction of cells in the normal background where the concentration of penicillin remains ineffective.

It was found that the same pattern developed on all penicillin plates treated with a given reagent, irrespective of the test organism, provided the proper concentrations of penicillin were used and provided the proper balance of the biological and physical factors involved in the cylinder plate method of assay was achieved. The latter was found to be largely a matter of properly controlling the relative lengths of the primary incubation period, when the organisms were in the lag period, and of the secondary incubation period, during which the organisms were in the log phase of growth and during which penicillin was diffusing through the medium in the plates.

The evidence indicates that penicillin affects aerobic gram-positive and gram-negative organisms through the same chemical systems. The threshold concentration at which its effects become manifest is, however, many times greater on plates seeded with gram-negative organisms than on those seeded with gram-positive organisms.

The proper use of trace amounts of cobalt lowers the effective threshold on test plates, a fact which may have practical clinical importance as well as theoretical interest, since the same phenomenon has been demonstrated *in vivo*.

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# LETHAL AND SUBLETHAL EFFECTS OF X-RAYS ON ESCHERICHIA COLI AS RELATED TO THE YIELD OF BIOCHEMICAL MUTANTS

RAYMOND R. ROEPKE AND FLORENCE E. MERCER

*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

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The isolation of growth-factor-requiring mutant strains of *Escherichia coli* from cultures exposed to X-rays during growth in a broth medium, and in one case from a culture transferred serially in broth without X-ray treatment, has been reported (Roepke, Libby, and Small, 1944). The yield of mutant strains was relatively low and variable, and the procedures would tend to limit the mutant strains to those able to grow at least as rapidly as the parent strain. Gray and Tatum (1944) and Tatum (1945) have reported the isolation of similar mutants from cultures of *E. coli* exposed for a short time to X-rays of high intensity. The X-rayed cultures were incubated 4 hours in a broth medium before plating for single-colony isolation. This procedure, although giving a relatively high yield of biochemical mutants, also tends to favor the isolation of those strains with the highest rate of growth.

This report deals with attempts to obtain biochemical mutants of *E. coli* by a procedure not affected by their relative growth rates and to determine some of the factors affecting the yield of mutant strains.

*Theoretical considerations.* From quantitative studies of the lethal effects of X-rays on *E. coli*, Wyckoff (1930) and Lea *et al.* (1936, 1941) concluded that death of a bacterial cell was the result of a single "hit" or absorption of a single quantum of X-ray energy in a vital structure in the cell. Lea and his coworkers (1941) suggested that these vital structures may correspond to genes and that death of a bacterial cell by X-rays may be considered as a lethal mutation. If this assumption is valid, the number of viable mutations produced by X-rays should increase in direct proportion to the number of cells killed by X-rays. On the basis of this assumption and with the further assumption that a viable mutation does not alter the sensitivity of the cell to X-rays, one may derive a relationship between the ratio of viable mutant to nonmutant cells and the survival ratio.

According to the "one-hit-to-kill" theory the log of the survival ratio decreases linearly with the time of X-ray treatment (Wyckoff, 1930). Thus,

$$(1) \quad \ln \frac{N}{N_0} = -kt \quad \text{or}$$

$$(2) \quad \frac{dN}{dt} = -kN,$$

where  $N_0$  = the initial number of viable cells,  $N$  = the number of surviving cells at any time,  $t$ , and  $k$  = a constant dependent on the intensity of the X-rays.

If  $x$  = the number of viable mutant cells of a particular type, i.e., cells unable to carry out reaction  $X$ ,  $n$  = the number of all other viable cells,  $n_0$  = the initial number of such cells, and  $R$  = the mean ratio of cells mutated (to mutant  $X$ ) to cells killed by X-rays, then the rate at which mutant cells of type  $X$  are killed =  $kx$ , the rate at which all other cells are killed =  $kn$ , and the rate at which mutant cells are produced =  $Rkn$ , from which

$$(3) \quad \frac{dx}{dt} = -kx + Rkn \quad \text{and}$$

$$(4) \quad \frac{dn}{dt} = -kn - Rkn.$$

Substituting from  $\frac{dx}{dt} = \frac{dx}{dn} \cdot \frac{dn}{dt}$ , equations 3 and 4 may be combined and integrated to give

$$(5) \quad \frac{x}{n} = \frac{K}{n^{(R/(1+R))}} - 1,$$

where  $K$  = the constant of integration.

If  $\frac{x}{n} = r$  when  $n = n_0$ , then  $K = (1 + r) \cdot n_0^{(R/(1+R))}$  and substitution in equation 5 gives

$$(6) \quad \frac{x}{n} = (1 + r) \cdot \left(\frac{n_0}{n}\right)^{(R/(R+1))} - 1.$$

According to this relationship the ratio of mutant cells of a specific type to all other cells surviving X-ray treatment varies in an inverse manner with the survival ratio. From the derivative of  $\frac{x}{n}$  with respect to  $n$ , it can be shown that

$\frac{x}{n}$  continues to increase without reaching a finite maximum as the survival ratio approaches zero. In other words, the greater the percentage of cells killed by X-rays, the greater will be the percentage of mutant cells among the survivors. If  $R > r$ , the value of  $x$  will pass through a maximum as the survival ratio decreases. However, in the case of biochemical mutants which cannot be isolated by a selective method one is interested in obtaining a high percentage of mutant cells in order to facilitate their isolation.

Equation 6 is dependent on the validity of the assumptions stated previously and on the use of experimental conditions that insure uniform exposure of the cells to X-rays. Since  $R$  in this equation is a ratio of probabilities with a variation around a mean value, the ratio  $\frac{x}{n}$  may be expected to show considerable experimental variation as the number of surviving cells approaches zero. Thus

the initial number of viable cells ( $n_0$ ) should be sufficiently high that at any desired survival ratio the number of surviving cells is appreciably greater than the value of  $R$ . Up to the range in which the number of double mutations becomes significant,  $x$  may be considered as the number of mutant cells of a general type, i.e., growth-factor-requiring cells.

Although equation 6 may be valid over an appreciable range of X-ray dosage, the experimental results indicate that it does not hold for more extensive X-ray treatment, the limitations being dependent to some extent on the experimental conditions.

#### METHODS

*E. coli* no. 15<sup>1</sup> was used in this study. Preparatory to X-ray treatment, single colony cultures were inoculated into the basal synthetic medium consisting of inorganic salts, glucose, and asparagine (Roepke *et al.*, 1944). In experiments involving "young" cells cultures in the basal medium were started with sufficiently large inocula to give visible turbidity and incubated until approximately half the maximum turbidity was obtained. The cultures were then cooled to 15 to 20 C to reduce the growth rate during centrifugation. "Old" cells were harvested from cultures started with a small inocula and incubated for 21 to 28 hours at 37 C, maximum growth being obtained within 18 hours. The harvested cells were washed once and resuspended in saline-phosphate buffer (0.50 per cent NaCl + 0.20 per cent  $\text{KH}_2\text{PO}_4$  adjusted to pH 7.0 to 7.2 with NaOH) to give viable counts of about  $1 \times 10^9$  cells per ml in the first two experiments and  $1 \times 10^9$  to  $7 \times 10^{10}$  cells per ml in subsequent experiments. Unless stated otherwise, X-ray treatment was initiated within 2 hours after preparation of the cell suspension.

In preliminary experiments the cell suspensions were X-rayed in the aluminum chamber used previously (Roepke *et al.*, 1944). The X-ray beam was admitted through a waterproofed cellophane window, and the suspension was stirred with a motor-driven glass stirrer. When it became evident that stirring alone was not sufficient to attain uniform exposure of the cells, the aluminum chamber was replaced with a pyrex test tube, 15 by 150 mm, the lower end of which was blown into a bulb 22 mm in diameter. The tube, containing 1.6 ml of cell suspension, was placed about 75 mm from the window of the X-ray tube and in such a position that the cross section of the X-ray beam more than covered the cross section of the cell suspension, including the walls of the pyrex tube several mm above its junction with the suspension. The suspension was stirred and the pyrex tube rotated continuously during X-ray treatment. The temperature of the suspension during incubation was maintained at 19 to 20 C in the first few experiments and at 10 to 14 C in the later experiments.

X-ray treatment was carried out with a General Electric X-ray diffraction unit, using a tube with a molybdenum target at 40 kv and 19 to 20 ma.

In the determination of the death curve samples of approximately 0.05 ml were removed with micropipettes at intervals of 15 to 45 minutes. The X-ray

<sup>1</sup> No. 9,723 of the American Type Culture Collection.

treatment was interrupted for 3 to 5 minutes during removal of the samples. The ability of a cell to grow into a visible colony on agar medium was used as the criterion of viability. Viable counts were made by plating with Difco nutrient agar and incubating 24 to 30 hours at 37 C.

The general procedure used in the isolation and identification of biochemical mutants has been described (Roepke *et al.*, 1944). Difco AC broth supplemented with a crude liver extract was used as the "complete" medium and will be referred to as AC broth. In some cases this was supplemented also with an autolyzate prepared from baker's yeast and sterilized by filtration. For single-colony isolation various dilutions of the X-rayed suspensions were flooded on the surface of AC agar (AC broth plus 2 per cent agar), and the excess was drained to one edge of the plate and removed with a pipette.

#### RESULTS AND DISCUSSION

In previous studies (Roepke *et al.*, 1944) in which cell suspensions contained in the aluminum chamber were exposed to X-rays, it was found that the decrease in the log of the survival ratio was essentially linear with time, although irradiation treatment was not extended beyond that giving a survival ratio of about  $10^{-3}$ . With more extensive X-ray treatment the plot of the log of the survival ratio versus the time of exposure deviated appreciably from linearity at low survival ratios (curve A, figure 1). In this experiment the samples were removed from the container without additional mixing. When the cell suspension was mixed with a pipette just prior to removal of the samples, the death curve was very irregular at low survival ratios, indicating that the motor-driven stirrer was not sufficient to insure uniform exposure of the cells.

The use of the pyrex tube in place of the aluminum chamber insured relatively uniform exposure of the cell suspension (curves B and C, figure 1). Some deviation from linearity is evident, but this is always in the direction of an increased death rate with time of X-ray treatment. The deviations from linearity in the first part of the curves obtained with suspensions of young cells (figures 1 and 2) are probably due to a discrepancy between plate counts and the number of viable cells. The cells harvested from actively growing cultures may occur in large part as short chains of incompletely divided cells (Robinow, 1945), so that many of the colonies obtained in plate counts may be derived from two or more viable cells. Thus, in the control suspension and in the early period of the X-ray treatment the actual number of viable cells would be higher than that indicated by plate counts, with the two values approaching equality as more cells are killed.

Curve D, figure 1, is a theoretical curve showing the relation between the log of the survival ratio, on the basis of plate counts, and the time of X-ray treatment when all of the cells exist in chains of four cells each with all of the cells initially viable. The calculations are based on the assumption that the death rate of individual cells follows equation 1.<sup>2</sup> As shown by the calculated curve E,

<sup>2</sup> On the basis of equation 1 the death of cells occurring in chains of 4 may be considered as analogous to a series of 4 consecutive, first-order reactions, as

figure 1, the percentage of colonies derived from more than one viable cell becomes negligible below a survival ratio of  $10^{-1}$  to  $10^{-2}$ . Thus, at lower survival ratios plate counts can be considered as an adequate indication of the number of viable cells. From the point of intersection of the linear portion of the experimental curves with the zero axis, it appears that the number of viable cells

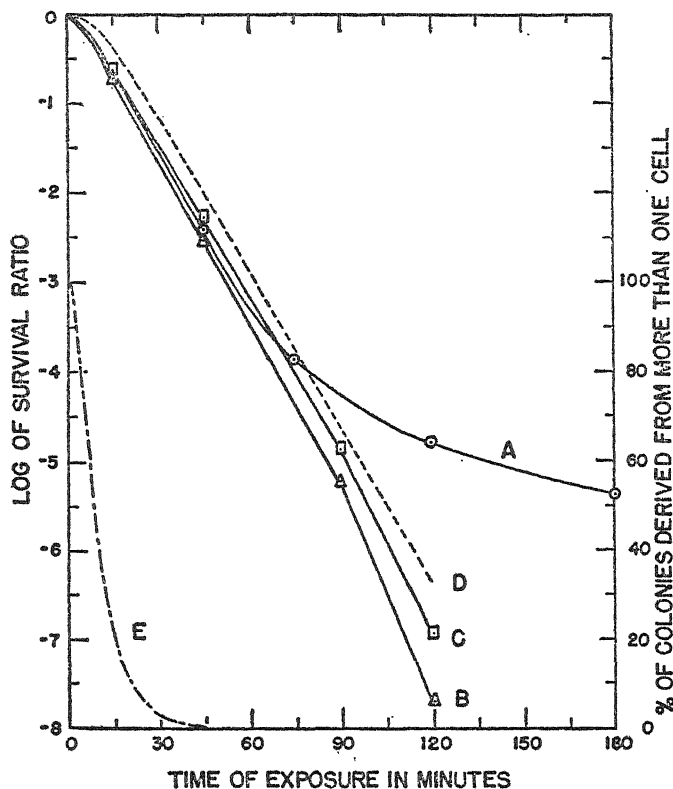
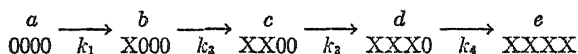


FIG. 1. X-Ray Treatment of Young Cells

A, suspension X-rayed in aluminum chamber; B and C, 2 portions of same suspension in pyrex tube, X-rayed immediately after preparation (B) and after storage at 4 C for 20 hours (C); D and E, theoretical curves of log of survival ratio as indicated by plate counts (D) and percentage of colonies originating from more than one viable cell (E), calculated on the supposition that all of the cells occur in chains of 4 cells each (see text).



where  $a$  = the number of chains in which all 4 cells are viable,  $b$  = the number of chains in which one cell has been killed, etc. Since the probability of killing any one of the viable cells in a chain is directly proportional to the number of viable cells in that chain, the rate constant  $k_1 = \frac{1}{3}k_2 = 2k_3 = 4k_4$ . The value of  $k_4$  (S/hour) was approximated from the relatively linear portion of one of the experimental, death rate curves. The viable count as indicated by plate counts corresponds to the sum  $a + b + c + d$ . The values of  $a$ ,  $b$ ,  $c$ , and  $d$  were calculated by means of the general equations used in calculating the variation with time of the quantity of decay products in a radioactive series (Rutherford *et al.*, 1930).

initially present in most of the suspensions of young cells was about twice that indicated by plate counts. This is in general agreement with the observations of Robinow (1945) on the structure of rod-shaped bacteria. In a few experiments the shape of the death rate curve indicated an appreciably greater discrepancy between plate counts and the number of viable cells (curve A, figure 2). This may have been the result of incomplete dispersion of the centrifuged cells.

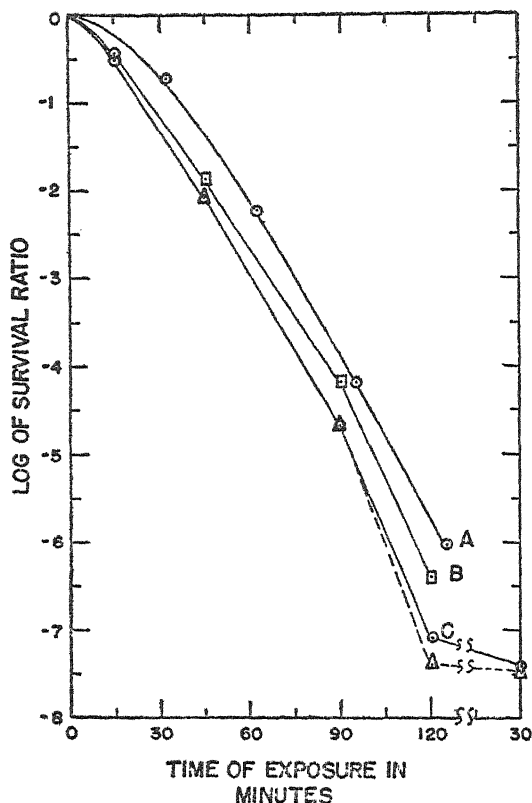


FIG. 2. X-Ray Treatment of Young Cells

A and B, cell suspensions X-rayed immediately after preparation; C, portion of same suspension as B X-rayed after storage at 4 C for 24 hours; —, plates incubated at 37 C, and - - - at 25 C. The last points on curves C obtained by keeping X-rayed suspension at 14 C for an additional 30 minutes before diluting and plating.

In two experiments the viable counts on the control (nonirradiated) suspensions increased 20 and 25 per cent during incubation at 20 C for 5 and 3 hours, respectively. However, this degree of growth or increased dispersion of the cells would not appreciably affect the shape of the death rate curve.

Death rate curves were determined on only two suspensions of old cells (figure 3). With one suspension, on which two runs were made after storage at 4 C for 18 and 22 hours, the change in slope in the initial portion of the curves is such as to indicate a heterogeneity of the cells with respect to X-ray sensitivity.

This was not investigated further to determine whether the heterogeneity resulted simply from storage of the suspension of old cells or from a spontaneous mutation affecting X-ray sensitivity in the culture from which the cells were harvested. However, storage of suspensions of young cells before irradiation did not result in a change in shape of the death rate curves (figures 1 and 2). A mutation resulting in increased resistance to ultraviolet and X-ray irradiation has been reported by Witkin (1946).

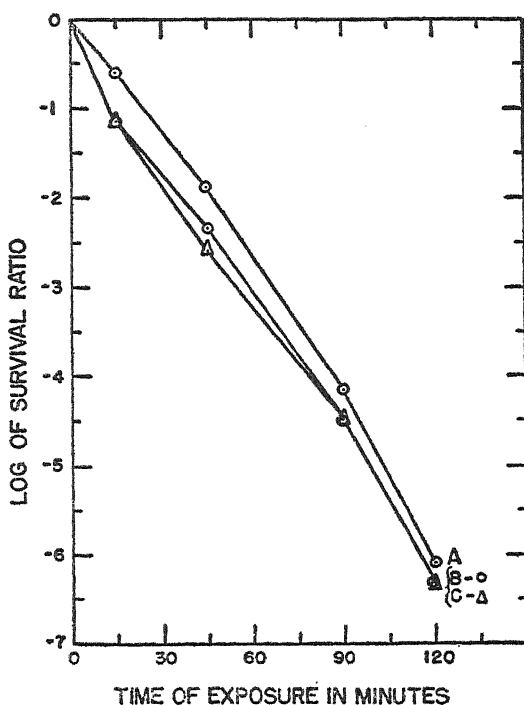


FIG. 3. X-Ray Treatment of Old Cells

A, cells harvested from 28-hour culture and X-rayed immediately; B and C cells harvested from 21-hour culture and X-rayed after storage at 4 C for 18 (B) and 22 hours (C).

The deviation of the death rate curve from linearity at low survival ratios (figures 1, 2, and 3) is probably due, in major part at least, to the cumulative effects of nonlethal hits in the less vital structures of the cells, resulting in increased fragility, or decreased viability, and finally death of the cells. This is indicated by the observations that the viable count of suspensions in the region of low survival ratios ( $10^{-6}$  or less) is dependent on the nature of the plating medium, on the interval between cessation of X-ray treatment and the time of plating, and on the temperature of incubation of the plates. Similar effects were observed by Hollaender (1943) following ultraviolet treatment of *E. coli* cells. It was found that the viable count of cells surviving ultraviolet treatment decreased during incubation in a phosphate buffer at a rate dependent on the

extent of ultraviolet treatment and the temperature of incubation. The surviving cells also showed a prolonged lag phase when incubated in broth.

Plate counts on X-rayed suspensions with survival ratios in the range of  $10^{-6}$  or less were found to be significantly lower (one-half to one-tenth) with AC agar than with Difco nutrient agar, and even lower counts were obtained with trypticase soy agar (Baltimore Biological Laboratories). On suspensions with higher survival ratios ( $10^{-5}$  or higher) the three media gave identical plate counts, within experimental error, although Difco nutrient agar gives appreciably slower growth and smaller colony size than the other two media. Mineral analyses of the three media (table 1) suggest an increased sensitivity of the X-rayed cells to

TABLE 1  
*The mineral composition of broth media*

	MILLIMOLES PER LITER					
	Na	K	Ca	Mg	Cl	PO <sub>4</sub>
Difco nutrient broth.....	8.25	5.37	0.0	0.46	2.34	2.96
AC broth.....	50.4	13.8	0.79	0.93	23.0	6.46
Trypticase soy broth.....	127	30.9				

TABLE 2  
*Effect of electrolyte concentration of plating medium on the viable count of a cell suspension X-rayed for 110 minutes*

AGAR MEDIUM	CONCENTRATION OF Na PLUS K	VIABLE COUNT*
	mM per liter	
Difco nutrient.....	13.6	46,400
80% AC†.....	51	23,300
AC.....	64	15,400
AC + KCl.....	101	5,320
AC + NaCl.....	152	1,620

\* Colony forming organisms per ml. The viable count on the nonirradiated suspension was  $3.03 \times 10^{10}$  cells per ml.

† AC agar diluted with sterile water.

salt concentration, since the viable count varied in an inverse manner with the concentration of electrolytes in the media. This is further indicated by the results of an experiment given in table 2. The addition of NaCl or KCl to AC agar resulted in a lower plate count on the X-rayed suspension, whereas dilution of the medium resulted in a higher count.

In one experiment the cells were washed and resuspended in dilute (0.005 M) phosphate buffer in place of the saline-phosphate buffer (0.086 M NaCl plus 0.015 M phosphate). Although the death curve was not determined, the survival ratio after 2 hours of X-ray treatment was  $10^{-9}$  or less (no colony-forming cells in 0.10 ml with  $2.2 \times 10^{10}$  viable cells per ml in the control suspension). Since similar treatment of cells suspended in the saline-phosphate buffer gave survival



ratios of  $10^{-6}$  to  $10^{-7}$ , it appears that the viability of X-rayed cells is decreased also when the electrolyte concentration of the suspending medium is reduced to a very low value. An increased sensitivity of the cells to electrolyte concentration may be due to increased permeability of the cells as a result of X-ray treatment. This is suggested by the studies of Ting and Zirkle (1940), who found that extensive X-ray treatment of blood produces a marked increase in permeability of the erythrocytes to potassium and sodium, resulting in swelling and finally lysis of the cells.

In two experiments in which suspensions of young cells were X-rayed for 120 and 140 minutes, several large clumps were noticed in the suspensions at the end of the treatment. In a third experiment in which a suspension was X-rayed for 125 minutes, the clumps appeared after incubation of the suspension in AC broth, with stirring, for 60 minutes. One or two large clumps appeared in each case with a large proportion of the cells remaining in suspension. The clumps were gelatinous in nature and difficult to disperse. Examination of stained films of such clumps showed a number of cells enmeshed in a homogeneously stained material, indicating that some of the cells had been lysed.

In the remaining experiments (X-ray treatment of 110 to 125 minutes) no clumping or agglutination was evident even when the X-rayed suspensions were stored for periods of 1 hour to several days. Thus, the decrease in viable counts observed during storage of the X-rayed suspension does not appear to be due, entirely at least, to an increased clumping of the cells. This is indicated also by the effect of the temperature of incubation of the plates on the viable count of X-rayed suspensions (curves C, figure 2).

Under our experimental conditions, then, the results indicate that down to a survival ratio of about  $10^{-5}$  the log of the number of viable cells decreases linearly with the time of X-ray treatment in accordance with the "one-hit-to-kill" theory of the bactericidal effect of X-rays. With more extensive treatment an increasingly greater proportion of the cells dies from the cumulative effects of "nonlethal" hits. In the latter range of X-ray dosage the ratio of cells mutated to cells killed would probably decrease, in which case equation 6 would not be valid. If a viable mutation does not alter the sensitivity of the cell to such cumulative effects, the yield of mutant cells would continue to increase with X-ray dosage but at a relatively slower rate. The data given in table 3, however, show that this may not be the case. The results of experiments 460 and 482 indicate that the mutant cells die at a greater rate than do the nonmutant cells during storage of the X-rayed suspensions. Cultures of the isolated mutant strains do not appear to differ significantly from the nonirradiated parent strain as regards viability or sensitivity to environmental factors, although this aspect has not been investigated in detail.

These results indicate that the ratio of mutant to nonmutant cells in the surviving cells may pass through a maximum in the range in which an increasing percentage of the cells dies from the cumulative effects of "nonlethal" hits. The yield of mutant cells in this range of X-ray dosage may be increased by the use of experimental conditions that permit a greater proportion of the injured

cells to grow into colonies on the complete medium. This may be accomplished by the use of more suitable suspending and plating media, by plating as soon as possible after cessation of X-ray treatment, and by irradiating with a higher intensity of X-rays. The latter would permit irradiation with a given dosage in a shorter time and hence would reduce the incidence of death of injured cells before the suspension is plated for single-colony isolation.

With X-ray treatment of *Neurospora*, Sansome, Demerec, and Hollaender (1945) found the percentage of mutants to increase with dosage without reaching a maximum over the range studied (to a survival ratio of  $10^{-4}$ ). With ultraviolet irradiation of fungi the percentage of mutant cells was found by Hollaender and Emmons (1941) and Hollaender *et al.* (1945) to pass through a maximum at a survival ratio of about  $10^{-2}$ . In contrast to our results with X-ray irradiation of bacteria, however, Hollaender and Emmons (1941) observed that with more extensive ultraviolet treatment the survival ratio and the yield of mutant cells were increased when the irradiated spores were incubated in a salt solution before plating.

Demerec (1946), in a study of X-ray-induced mutations to virus resistance in a strain of *E. coli*, found that the yield of virus-resistant cells or colonies was increased appreciably when plates of the irradiated cells were incubated for several hours before applying the virus as a selective test for the mutant character. From this one might expect an increase in the yield of growth-factor-requiring mutants if the irradiated cells were incubated for a time in a broth medium before plating for single-colony isolation. This would not be true, however, if the results obtained by Demerec were due only to a delay in the manifestation of the mutation with all of the descendants exhibiting the mutant character. This has been suggested as a possibility by Demerec and appears to be substantiated by the results of experiment 472 (table 3), in which it was found that incubation of the X-rayed cells in AC broth before plating failed to increase the percentage of biochemical mutants. The data, however, are not sufficient to warrant a definite conclusion.

Although the data given in table 3 serve to illustrate the yield of biochemical mutants that can be obtained with X-ray irradiation, they are inadequate to demonstrate a relation between the yield and survival ratio since all of the variables were not adequately controlled. The time of plating varied from about 15 to 45 minutes after cessation of X-ray treatment, and the plates were incubated at temperatures varying from 20 to 37 C. In experiments 495 and 546 the X-ray intensity, as indicated by the death rate, was about half that used in other experiments. In experiment 468 the cell suspension appeared to be heterogeneous as regards sensitivity to X-ray radiation (curve B, figure 3). Relatively few colonies were available for isolation in some experiments owing to failure to pour a sufficient number of plates with the proper dilution of the X-rayed suspension.

The classification of mutant cultures (table 3) is somewhat arbitrary. Most of those classified as unstable or questionable are so unstable as to make identification of the growth requirements difficult or uncertain, whereas others appear

to have complex requirements and may be contaminants. Some of the strains listed as stable mutants are relatively unstable, although the growth factor requirements could be readily determined. The number of different mutant strains can be considered only as the minimum number, since the mutations can be differentiated only on the basis of known differences in the growth requirements. Although identical mutant strains may have been isolated from the

TABLE 3

*The yield of biochemical mutants obtained by X-ray irradiation of suspensions of E. coli*

EXPERIMENT NO.	AGE OF CULTURE	SURVIVAL RATIO	NUMBER OF COLONIES ISOLATED		NUMBER OF MUTANT CULTURES		
			Plated immediately*	Plated after storage	Stable		Unstable or questionable
					Total	Different strains	
450	young	$1/3.7 \times 10^6$	16	—	1	1	0
460	young	$1/1.4 \times 10^6$	1,090	—	14	10	2
		$1/1.4 \times 10^6$	—	911†	0	0	1
462	young	$1/8.7 \times 10^4$	1,028	—	20	13	3
482	young	$1/1.3 \times 10^6$	329	—	8	8	1
		$1/1.3 \times 10^6$	—	125‡	0	0	0
489	young	$1/6.5 \times 10^5$	926	—	13	11	1
495	young	$1/1.5 \times 10^4$	300	—	0	0	0
546	young	$1/2.7 \times 10^5$	311	—	1	1	0
468	old	$1/2.1 \times 10^6$	232	—	1	1	0
		$1/2.1 \times 10^6$	—	55§	0	0	0
472	old	$1/2.2 \times 10^6$	188	—	3	3	0
		$1/2.2 \times 10^6$	—	100	0	0	0
Totals	young		4,000	—	57	28	11
			—	1,036	0	0	1
	old		420	—	4	4	0
			—	55	0	0	0

\* Irradiated suspension plated within 45 minutes after cessation of X-ray treatment.

† Suspension diluted in saline buffer and stored at 4 C for 1 to 8 days.

‡ Suspension diluted in AC broth and stored at 4 C for 15 hours.

§ Suspension diluted in saline buffer and stored at 4 C for 24 hours.

|| Suspension diluted in AC broth and incubated at 37 C for 3 hours, resulting in a reduction in the viable count of 71 per cent.

same irradiated suspension, it is likely that such mutations arose independently, since there was little chance for a mutated cell to divide before the suspension was plated on the agar medium. One of the mutant strains isolated from irradiated suspensions of old cells differed from any of those obtained from young cells.

Only 10 contaminant colonies were obtained from a total of 5,611 isolated colonies. Eight of these were obtained in one experiment (no. 482) and were identical in morphology. A colony was considered as a contaminant if the cells

differed appreciably from *E. coli* both in morphology and growth requirements. None of the strains considered to be mutant differed noticeably in morphology from *E. coli* with the possible exception of a strain requiring thymine. The cells of this strain were considerably elongated or occurred in long chains when grown in limiting concentrations of thymine nucleotide. The characteristics and growth requirements of the mutants obtained in these experiments will be described in subsequent reports.

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#### SUMMARY

Suspensions of *Escherichia coli* were irradiated with X-rays under conditions which insured essentially uniform exposure of the cells. The log of the survival ratio was found to decrease linearly with the time of irradiation, in accordance with the "one-hit-to-kill" theory of the bactericidal effects of X-rays, down to a survival ratio of about  $10^{-5}$ . A deviation from linearity in the initial portion of the death rate curve is considered to be the result of a discrepancy between plate count and the number of viable cells.

With more extensive X-ray treatment the death rate increases, apparently as the result of the cumulative effects of "nonlethal" hits, which render the cells more fragile or more sensitive to environmental conditions. Viable mutations appear to result in an increased sensitivity to such cumulative effects as indicated by the effect of storage of the X-rayed suspensions on the yield of biochemical mutants.

A total of 61 growth-factor-requiring mutant cultures, consisting of at least 29 different strains, were obtained from 4,420 colonies isolated from plates poured shortly after X-ray treatment of resting cell suspensions. No mutants were obtained from 1,091 colonies isolated from plates poured after storage of the X-rayed suspensions at 4 C for 15 hours to 8 days.

The results of this study illustrate some of the factors to be considered in a quantitative study of X-ray-induced mutations or in an attempt to obtain high yields of biochemical mutants.

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# MUTATION AND ADAPTATION OF PHYTOMONAS STEWARTII<sup>1</sup>

RALPH E. LINCOLN

*Department of Botany and Plant Pathology, Agricultural Experiment Station, Purdue University, Lafayette, Indiana*

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Bacteriologists often employ procedures such as growing a culture of bacteria at high temperatures in immune serum or in  $\text{LiCl}_2$  to stimulate variation in the culture. However, the mere observation of variation after such treatment is not proof that the treatment caused the variation to originate. It seems equally probable that in the new or changed environment a spontaneous mutation may occur and this variant may be better adapted than the parental type. If this were true, a complete replacement of one type by another could occur without the treatment having changed the rate of mutation.

Except for rare cases of extranuclear inheritance, inherited variation, at least with the higher organisms, originates either by mutational changes in existing genes and chromosomes or by new gene combinations, such as occur after gametic fusion with subsequent segregation, somatic segregation, or heteroploidy. The problem of gametic fusion using genetically distinct parental types as it relates to bacterial variation remains unstudied except for negative experiments by Sherman and Wing (1937) and by Gowen and Lincoln (1942).

Several workers have noted that the origin of bacterial variation appears to be mutational, since variation is shown to be at random, and that variation of one character does not necessarily cause variation in other characters. Critical evidence of the mutational nature of bacterial variation has been obtained from two lines of approach. Zelle (1942) has demonstrated discontinuous variation as regards colony type of *Salmonella typhimurium*, using a modified Chambers single cell technique. Lincoln and Gowen (1942) have shown that, after treatment of *Phytomonas stewartii* (Smith) Bergey *et al.* with X-rays, variation is observed that is similar to the natural variation that occurs during growth in broth, but at rates as much as 600 times that of natural variation. Indirect evidence of the mutational origin of many kinds of bacterial variants has been obtained by these and numerous other workers. After genetic variation occurs in a pure stock, either by mutation or by new gene combinations, the population is a mixture of genotypes, and that population becomes subject to selective forces that had no differential effect while all individuals were genetically identical. Though numerous cases could be cited in which there is a change in a culture from one characteristic type to that of a contrasting type, there are few actual data indicating recognition of selection as a force affecting bacterial populations.

It is the purpose of this paper to present evidence on the effect of temperature in changing the rate of variation and to determine the stability of bacterial

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populations of *P. stewartii* in different environments, both from the standpoint of pure cultures of a single type and of known mixtures of cultures of differing types.

#### EXPERIMENTAL MATERIALS AND METHODS

Two cultures of *P. stewartii* and variants of each stock were used in these experiments: stock 400 produces a small, compact, dry, highly colored colony on nutrient glucose agar; stock 500 produces a large-spreading, mucoid, nonpigmented colony. All stocks used were plated repeatedly (5 to 7 times) from isolated single colonies. With this species McNew (1938) has shown that a high percentage of the colony loci on poured agar plates are seeded with single cells. After 3 or more platings the probability is tremendously high that at least once in this process the colony picked originated from a single cell.

To observe the rate of mutation bacteria from a single colony were seeded into test broth and allowed to grow for the desired time. With nutrient broth at 24 C this period was 24 hours, during which approximately 16 generations occurred. After growth the broth culture was vigorously and repeatedly shaken over a period of half an hour to obtain as many bacteria as possible occurring as individual cells. A dilution was then made so that one drop of liquid would contain the approximate number of bacteria desired per plate. In all cases it was desired to obtain mature colonies, individual and distinct from one another, so that colony characteristics could be easily observed. One drop of broth of the desired dilution was placed on the surface of a hardened agar plate and smeared evenly over the entire surface of the plate by means of an L-shaped rod. Plates were incubated at room temperatures for 48 hours, then observed at 9X magnification for colony classification. Reflected light at 75 degrees from the lens was used. The mutation rate was calculated by formula 2 of Demerec and Fano (1945):

$$r = 3.32 aN \log_{10} \frac{CaN}{0.693}$$

in which

$r$  = likely average number of mutant bacteria per culture,

$C$  = number of cultures,

$N$  = average number of bacteria in  $C$  cultures,

$a$  = the mutation rate per division cycle.

Their published constant of 1.6 is here corrected to 3.32. It is apparent from the values published in table 3 of their paper that they must also have used the constant 3.32. This formula is a modification of equation 8 of Luria and Delbrück (1943), and these authors have discussed the assumptions on which the derivation of this equation is based. Incorrectness of any of these assumptions tends to make the calculated mutation rate greater than the real rate. It is probable that the actual mutation rate is greater than the calculated rate since factors such as mutant cell lethality, lowered viability, slower generation time, a possible chromatin segregation mechanism, inability to observe small changes, and random loss of variants by using small samples from a population all tend to keep the number of mutations observed at a minimum.



Essentially the same technique of sampling and plating has been used to determine population changes due to selection. With the mixed cultures technique only variants widely different from each other as regards colony characteristics were used.

#### EFFECT OF TEMPERATURE UPON THE RATE OF MUTATION

Growth of *P. stewartii* occurs in nutrient broth between the temperatures of approximately 10 and 38 C. Five temperatures within this range were selected to determine how the rate of mutation is affected by growth temperature. These temperatures were 12, 18, 24, 30, and 36 C.

Bacteria of a single colony were suspended in nutrient broth, and after repeated shaking five identical cultures were made in which the test broth contained approximately 3,000 bacteria per ml. At this time platings were made to determine variation initially present. One tube was then incubated at each of the five test temperatures. Since bacterial growth occurred at different rates at the different temperatures, platings were made to estimate both the number of bacteria per ml of broth and the variation present after approximately the same amount of growth had occurred in each culture as indicated by faint clouding of the broth cultures. The amount and rate of mutation occurring in these experiments has been summarized in table 1.

The observed variation in the original inoculum was zero. This low variability was obtained, after preliminary experiments, by maintaining stocks at low temperatures and by picking a colony to use as the initial inoculum from an agar plate containing only typical colonies. As shown in table 1, the mutation rate increases as growth temperature increases, both as regards the number and the kinds of mutant colonies observed. In the average tube at 12 C variability was rare; however, several different variants, often in large numbers, were observed in the average tube at 36 C. The fact that a sample from each colony population was grown at each of the five temperatures makes the observed trend for mutation to increase as temperature increases very significant.

Stock 401 originated as a mutant of stock 400 and appears identical with the parent in all respects except for the change from dark yellow to pale yellow colony color. The mutation rate of the pale yellow stock is lower at all temperatures than the mutation rate of the dark yellow stocks, and this difference is highly significant when one stock is compared with the other at all temperatures. It is apparent that some change in the genic balance has occurred because of the mutation of the dark yellow locus to pale yellow, resulting in greater genetic stability. It is probable that each mutation affects the genic balance and usually has pleiotropic effects on the organism.

The differential effect of temperature on mutation of specific characters is particularly striking. The rate of mutation from dark yellow to pale yellow is changed significantly between the temperatures of 24 and 30 C, being relatively stable at the temperatures of 24 or less and becoming increasingly mutable at 30 and 36 C. The changes from dark yellow to white or from pale yellow to white also are increased at the temperatures of 30 to 36 C. All these changes are from a darker color to one of less intense color. The change from pale to dark

TABLE 1  
Mutation rate of *Phytonomas stewartii* grown at five temperatures

BACTERIAL STRAIN	TEMP.	CUL- TURE UNIT	COL- ONIES OB- SERVED	BACT. CUL- TURE	MUTANT COLONIES OBSERVED												Sector	Total
					Dark yellow	Pale yellow	White	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	Smooth	Small	Pale yel., R <sub>4</sub> smooth	White, R <sub>2</sub>	Un- Stable		
	C	No.	× 10 <sup>3</sup>	Agg × 10 <sup>6</sup>														
Stock 400				78														
Cultures mutating.....	36	11	135		—	11	8	6	4	4	2	6	3	—	—	—	2	
Mutant colonies.....					—	1,074	23	28	61	8	4	306	4	—	—	—	1,516	
Mutation rate × 10 <sup>-7</sup> .....					—	3,460.0	101.0	114.0	239.0	36.1	19.3	1,500.0	19.3	—	—	—	4,340.0	
Stock 400				105														
Cultures mutating.....	30	11	192		—	11	6	3	4	2	2	1	3	—	—	—	6	
Mutant colonies.....					—	315	66	6	30	5	6	1	3	—	—	—	441	
Mutation rate × 10 <sup>-7</sup> .....					—	953.0	229.0	25.9	106.0	22.4	25.9	7.6	14.2	—	—	—	1,300.0	
Stock 400				109														
Cultures mutating.....	24	11	224		—	8	5	3	1	1	—	2	3	—	—	—	3	
Mutant colonies.....					—	27	8	10	1	2	—	5	3	—	—	—	60	
Mutation rate × 10 <sup>-7</sup> .....					—	84.0	29.1	35.5	4.6	8.7	—	19.1	11.7	—	—	—	180.1	
Stock 400				96														
Cultures mutating.....	18	11	356		—	8	5	2	2	1	1	2	3	—	—	—	5	
Mutant colonies.....					—	24	16	4	2	2	1	10	3	—	—	—	72	
Mutation rate × 10 <sup>-7</sup> .....					—	62.7	30.8	10.9	5.8	5.8	3.2	24.0	8.0	—	—	—	142.0	
Stock 400				72														
Cultures mutating.....	12	11	416		—	5	3	2	—	—	2	1	1	—	—	—	—	
Mutant colonies.....					—	14	4	2	—	—	4	1	1	—	—	—	28	
Mutation rate × 10 <sup>-7</sup> .....					—	29.2	9.6	6.3	—	—	9.6	2.9	2.9	—	—	—	54.1	
Stock 401																		
Cultures mutating.....	36	5	129	83	2	—	4	2	—	1	1	1	1	—	—	—	—	
Mutant colonies.....					4	42	6	6	—	1	1	3	1	—	—	—	60	
Mutation rate × 10 <sup>-7</sup> .....					26.5	223.0	36.3	36.3	—	7.8	7.8	20.7	7.8	—	—	—	308.0	
Stock 401				116														
Cultures mutating.....	30	5	117		1	—	5	—	1	—	—	3	—	—	—	—	—	
Mutant colonies.....					2	—	14	—	2	—	—	2	—	—	—	—	94	
Mutation rate × 10 <sup>-7</sup> .....					15.0	—	88.0	—	15.0	—	—	34.2	—	—	—	—	137.0	
Stock 401				107														
Cultures mutating.....	24	5	119		2	—	2	1	—	—	—	3	1	—	—	—	—	
Mutant colonies.....					5	—	6	1	—	—	—	4	2	—	—	—	18	
Mutation rate × 10 <sup>-7</sup> .....					34.1	—	40.1	8.4	—	—	—	28.0	14.6	—	—	—	108.0	
Stock 401				88														
Cultures mutating.....	18	5	62		—	—	1	—	—	—	—	1	1	—	—	—	—	
Mutant colonies.....					—	—	14.9	—	—	—	—	1	1	—	—	—	4	
Mutation rate × 10 <sup>-7</sup> .....					—	—	—	—	—	—	—	14.9	14.9	—	—	—	51.3	
Stock 401				76														
Cultures mutating.....	12	5	140		1	—	1	—	1	—	—	—	—	—	—	—	—	
Mutant colonies.....					1	—	7.5	—	2	—	—	—	1	—	—	—	5	
Mutation rate × 10 <sup>-7</sup> .....					7.5	—	—	—	13.6	—	—	—	7.5	—	—	—	30.4	

yellow colony color was not affected by temperature. With similar stocks Lincoln (1940) has shown that at room temperatures the pattern for natural mutation of the color genes follows the same general pattern as that found in these experiments, and working with white stocks derived by mutation from dark yellow ones he did not observe the mutation from uncolored to colored colony type. These observations could indicate a multiple allelic series of genes. The mutations to smooth, rough 2, and rough 3 occur at very high rates at the higher temperatures. Other characters change with greater frequency at high growth temperatures than at low, but the increase in mutation rate with these other characters approximates a van't Hoff curve. Working with *Drosophila*, Plough

TABLE 2

Percentage of frequency of spontaneous mutations during growth at different temperatures

	DARK YELLOW STOCK TEMPERATURE					TOTAL	PALE YELLOW STOCK TEMPERATURE					TOTAL	P.* OF TOTAL
	36	30	24	18	12		36	30	24	18	12		
Dark yellow to pale yellow (or reciprocal)	70.2	71.1	45.0	32.0	50.0	68.3	6.5	8.0	27.8	0	20.0	10.4	0.01†
Dark yellow (or pale yellow) to white.....	1.5	14.9	13.4	21.4	14.3	5.5	68.8	56.0	33.3	25.0	20.0	55.7	0.005†
Rough 1 (normal) to other roughs.....	6.5	10.6	21.6	11.9	21.4	8.3	13.5	8.0	5.6	0	40.0	11.3	>.05
Rough 1 (normal) to smooth.....	20.8	0.2	8.3	13.3	3.6	15.2	4.8	20.0	22.2	25.0	0	11.3	>.05
Normal colony size to small.....	0.2	0.7	5.0	4.0	3.6	0.7	1.6	0	11.1	25.0	20.0	4.4	.008†
Normal colony to sectored.....	0.2	1.4	5.0	9.4	0	0.9	1.6	0	0	25.0	0	1.7	>.05
Stable (normal) to unstable forms.....	0	0.2	1.7	0	0	0.1	0	0	0	0	0	0	>.05
Multiple mutations.....	0.6	0.9	0	8.0	7.1	1.0	3.2	8.0	0	0	0	3.5	>.05

\* "P." indicates probability.

† Indicates that the two stocks differ by odds of 99:1 or greater.

and Ives (1935) observed that heat tends to induce mutation in some genes more than in others.

It has been shown that the two stocks differed significantly in the rate of mutation. How nearly alike the two stocks are in their pattern of mutation is shown in Table 2, in which the percentage of the relative frequency of different mutations is given. Mutation from rough 1 colony type to other rough types or to smooth colony type and the occurrence of double mutations, of sectored colonies, or of unstable forms occur with nearly equal frequency in the two stocks, as indicated by a probability greater than 0.05 for the total on the five observations on each stock being alike. The change from dark yellow to pale yellow, however, occurs with greater frequency than does the reverse change, and the frequency of mutation from pale yellow to white occurs at a greater rate than

does the mutation from dark yellow to white. These observations are probably related and would be expected if the factors for dark yellow, pale yellow, and white colony color were part of a multiple allelic series of genes. The occurrence of small colony type in the pale yellow stock also is higher than in the dark yellow stock.

Considering the mutation rate at 12 C as the control, the temperature coefficient ( $Q_{10}$ ) for mutation in the dark yellow stock is approximately 5.0, whereas in the pale yellow stock the coefficient is about 2.5. Plough (1941) observed that mutation frequency in the first and second chromosome of *Drosophila* had a temperature coefficient in the neighborhood of 5.0, whereas Muller (1928) observed a  $Q_{10}$  between 2 and 3 for mutation in *Drosophila*.

TABLE 3

*Percentage of dark yellow type colonies when an unstable dark yellow stock segregating for dark yellow and pale yellow colony type was grown at three temperatures*

DARK YELLOW COLONIES										
Colony	Initial	33 C			24 C			15 C		
		24 hr	72 hr	96 hr	24 hr	72 hr	96 hr	24 hr	72 hr	96 hr
	%	%	%	%	%	%	%	%	%	%
1	40	52	31	11	70	62	60	83	80	82
2	38	31	17	6	34	44	42	35	65	58
3	48	31	24	16	36	37	40	62	60	61
4	54	32	20	21	57	50	46	56	67	72
5	43	28	22	14	47	51	50	53	69	64
6	25	25	15	11	37	40	38	44	49	51
7	73	31	20	7	58	62	58	67	74	70
8	70	27	22	15	61	58	55	74	75	76
9	65	40	15	7	54	58	56	64	67	74

Work with an unstable, dark yellow mutant found in the smooth mucoid stock 500 adds evidence to the increased lability of the dark yellow color gene at high growth temperatures. This unstable variant segregated for both dark and pale yellow colonies. On serial plating of individual colonies it was discovered that all the pale yellow type colonies were stable, whereas the dark yellow type continued to throw a variable percentage of pale yellow colonies on successive platings. Each color type appeared identical, except for color, when grown on nutrient glucose agar at room temperature; but when the plates were incubated at 30 C, all pale yellow colonies were normal in size and appearance, but the dark yellow ones were relatively very small and ridged. A high proportion of the dark yellow colonies contained pale yellow sectors that appeared as typical "bursts."

Dark yellow colonies were suspended in nutrient glucose broth. Aliquots of the same colony were grown in nutrient glucose broth at 3 temperatures. The change in proportion of the two types observed is given in table 3. These data show that after 24 hours' growth at high temperature the dark yellow type has

been partially replaced by the pale yellow type. At 24 C both types grow well, and there is no definite trend for one type to replace the other. At 15 C, however, there is an increase in the proportion of dark yellow types at the 72- and 96-hour sampling, although this trend may not be apparent in the initial stages of growth. The nature of the instability observed in this stock is not known, but since it was impossible to obtain a dark yellow stock that remained pure for color, whereas a pure culture of the pale yellow type could be obtained, it is believed that this stock contained a mutable gene that became more labile as the temperature increased. These observations would indicate that the mutable dark yellow gene became highly mutable at 33 C as compared to temperatures of 24 C or lower.

TABLE 4

*Summary of rate of mutation of stock 400 and of six mutant stocks derived from it when grown on nutrient broth at 24 C, with calculation of time needed for mutation to effect a given change in the population*

STOCK	COLONY		TUBES TREATED AS UNITS	COLONIES OBSERVED		MUTATION RATE	GENERATIONS NEEDED FOR MUTATION AT OBSERVED RATE TO EFFECT CHANGE TO MUTANT TYPE	
	Color*	Type†		Total	Mutant		1% change ( $q_1 = 0.99$ )	50% change ( $q_1 = 0.5$ )
				$\times 10^3$		$\times 10^{-7}$		
400	Dy	R1	11	224	260	183.0	548	38,200
428	Dy	R4	8	95	32	196.0	505	35,200
441	Dy	S	8	162	96	388.0	257	17,800
491	Wh	S	8	78	5	51.2	1,921	135,000
435	Py	S	8	145	17	2.4	4,100	288,000
446	Py	S	8	212	13	1.2	8,200	576,000
427	Py	R2	8	137	27	133.0	742	52,000

\* Dy = dark yellow; Py = pale yellow; Wh = white.

† R = rough; S = smooth.

#### IMPORTANCE OF MUTATION AND SELECTION IN EFFECTING CHANGES IN BACTERIAL POPULATIONS

When maintained as stock cultures, most mutants of *P. stewartii* were stable, and variant colony types were seldom found in these stocks even after being maintained by routine procedures for several months. Occasional mutants were found, however, that were difficult to maintain as a stock culture because of the occurrence of a high percentage of undesired colony types, either of the parental type or of some other variant types. Such instability could be due to a very high rate of mutation, to mutation with subsequent selection of the mutant, or to still other factors. To test these possibilities six mutant stocks of strain 400 were selected on which to determine the mutation rate and the mutant's ability to compete with its parental stock in nutrient glucose broth.

*Importance of mutation.* Mutation rates of the six selected mutants and of the parental stock (400) are given in table 4. These rates were determined after

24 hours' growth in nutrient broth at 24 C. There is considerable variation among these rates. Such variation is not unexpected if mutation is considered random in nature, particularly since the determination of each rate is based on a relatively small number of colonies.

To readily observe the change from a culture of one type of organism to a mutant type necessitates a mass change in that population. For example, in the mutation studies of culture 441 an average of about 1,700 colonies would need to be examined before a mutant colony would be observed; yet after 441 is maintained as a stock culture for 3 months (3 mass transfers each followed by 48 hours' growth at room temperature and storage for 30 days in a refrigerator at 7 to 10 C), it is not unusual to observe more than 50 per cent of the colonies of a mutant type. Can mutation of the order observed for these stocks effect such a mass change in a bacterial population?

The time required for mutation to change one type to some determined proportion of mutant types can be calculated. If

$q$  = proportion of mutant type at time  $t$ , and

$u$  = mutation rate,

then the shift in  $q$  due to mutation is

$$\frac{dq}{dt} = -uq$$

$$-t = \frac{\ln q}{u} + C$$

$$q = \frac{1}{e^{-u(t+c)}}$$

$$\frac{q_1}{q_0} = \frac{e^{-u(t_1+c)}}{e^{-u(t_0+c)}}$$

$$\ln \frac{q_1}{q_0} = u(t_0 - t_1)$$

When  $t_0 = 0$

$$t_1 = -\frac{1}{u} \ln \frac{q_1}{q_0}$$

The average rate of all mutation in the parental stock 400 is about  $1.81 \times 10^{-8}$  (table 4). The time required for mutation to change 50 per cent of the cells of the culture from parental type to mutant type would be  $t = -\frac{1}{0.000,018} \ln \frac{0.5}{1.0}$  or 38,200 cell generations. With this organism about 2,120 days of growth in the logarithmic growth phase would be required for this number of generations to occur. The number of generations required for mutation to effect a change from the parental type to 1 per cent and to 50 per cent of mutant types for each of the stocks studied is included in table 4.

If reverse mutation (from mutant back to normal type) were considered,

the time required for this change to occur would become even greater. All stocks seem very stable when considered in terms of the number of generations required for mutation to effect an observable change of type in a culture. Some influence, other than mutation, must be acting on a population to account for the change of type observed to occur in cultures such as 441 when carried as stock cultures.

*Selection within mixed populations.* If known proportions of two readily differentiable strains of bacteria were mixed together and the proportions of each type followed by subsequent platings, the interaction of one type with the second could be measured. A change in the relative frequency of one type of individuals

TABLE 5

*Changes in bacterial populations when known proportions of the mutant and parental stocks are grown together*

MUTANT	PROPORTION OF PARENTAL TYPE COLONIES (STOCK 400) AFTER INDICATED DAYS OF GROWTH										
	0	1	2	3	4	6	8	10	12	14	17
	%	%	%	%	%	%	%	%	%	%	%
427	39	43	54	52	64	73	92	97	99	98	98
428	17	16	23	54	44	87	94	99+	99	100	99+
435	60	51	56	62	48	76	87	87	93	94	99+
441	33	68	87	92	93	97	99	97	100	99	99+
441	29	23	90	99	99+	99+	100	100	100	—	—
446	71	89	99	99	100	100	—	100	—	—	—
491	53	64	73	87	89	97	91	95	97	96	98
491	49	54	44	70	70	86	92	86	64	97	97

in respect to the second type would indicate selective growth, and the intensity of selection could then be measured.

The mutants used in the preceding section were initially selected to be readily differentiated from the parent strain (400) by their colony morphology on agar. Each mutant was mixed with stock 400 and the proportion of each type followed by plating at desired intervals of time after growth in nutrient broth at 24 C. Data for these platings are given in table 5. No mutant tested grew better than did 400, the parent stock. Relative competitive ability of the various mutants is roughly indicated by the rate at which change in the two types occurs. When stock 400 was mixed with any of the variants tested in this experiment, replacement of the variant type by the parental 400 stock was generally rapid and in two cases complete. The average proportion of the parental type present in the initial inoculum was 47 per cent, and after 10 days' growth it had increased to 94.5 per cent. This change occurred in less than 180 cells generation. For

mutation to effect this change at the rate  $u = 0.0001$  would require 5,560 generations, as determined by the formula developed in the preceding section. Obviously individuals of strain 400 are reproducing more rapidly than those of the mutant stocks, thereby increasing the comparative frequency of the 400 genotype. Selection in a bacterial population may be considered equivalent to genic selection, as discussed by Wright (1931), if one assumes that bacteria are asexual organisms dividing by mitosis.

As shown in table 5, selection pressure is low until the logarithmic phase of growth is over, then selection pressure increases very markedly. Also it is apparent that selection pressure against the different mutants varies. This is shown in other selection experiments summarized in table 6, in which growth in several environments is considered. Stocks adapted in one environment

TABLE 6  
*Proportion of four bacterial types present after growth in mixed culture*  
Plated at 48-hour intervals; data average value of 2 tubes

ADDITIONS TO NUTRIENT BROTH	TEMP.	HOURS OF GROWTH	PROPORTION OF RESPECTIVE TYPE				DEATH OF CULTURE OBSERVED
			500*	520†	400‡	427§	
—	C	None	%	%	%	%	hours
None	24	606	54	25	19	2	>798
1% glucose	12	798	100	0	0	0	>798
1% glucose	24	462	1	0	27	72	500
1% glucose	36	272	0	0	100	0	300
1% glucose + 5% NaCl	24	798	0	100	0	0	>798
1% lactose	24	798	0	0	52	48	>798
10% glucose	24	366	47	0	44	9	>798
			78	4	20	2	375

\* Large, mucoid, smooth, yellow colony.

† Small, mucoid, smooth, white colony. Mutant of 500.

‡ Small, rough, type 1, nonmucoid, dark yellow colony.

§ Rough, type 2, nonmucoid, pale yellow colony. Mutant of 400.

may be entirely unadapted in a second environment. This phenomenon is shown best by stock 520, which in these mixtures was unable to compete in any environment except 36 C. This temperature is near the maximum at which growth will occur.

#### DISCUSSION

The origin of variation and the interaction of the variant with the parental type are distinct and separate problems of bacterial variation so closely interrelated that it is difficult to separate one from the other. It is recognized that in this study the two problems have not been entirely separated, but the methods used have allowed little possibility for one to influence the other. A formula to determine the number of generations for mutation to effect a certain change in a population has been developed. It has been shown that mutation alone is so infrequent as to be ineffective in causing a rapid mass change in a population.



By its very infrequency mutation can be ignored as a factor influencing selection except when the proportion of one type is near zero or one. In the determination of the mutation rate by the method used selection may be ignored, since selection has been shown to be relatively ineffective in changing the frequency of a genotype until the logarithmic phase of growth has been exceeded. In determining each of these factors—mutation or selection—small corrections could be made for the factor not studied. However, to do so would necessarily complicate the formula developed and in these cases would not affect the conclusions drawn.

Mutation and selection have been shown to be two very important factors in the evolution of bacterial populations. Mutations occur during growth at rates of the order observed in higher organisms. Although the nature of bacterial inheritance is still uncertain, the heritable material or genes must be duplicated and divided before cell division takes place. When the parental genes are not exactly reproduced in two daughter cells, mutation occurs.

This study has shown approximately a 10-fold increase in mutation rate when two stocks of *P. stewartii* were grown at a temperature of 36 C as compared with 12 C. Intermediate growth temperatures had an intermediate effect on mutation rate. It is interesting to note that the physical forces that influence mutation in high organisms are also very effective in changing mutation rate in bacteria.

Once variation is achieved, whether by mutation in a pure culture or by mixture of types, selective forces may act upon the different genotypes. In these experiments the ability of six different mutants to compete with their parent stock was determined. In nutrient broth none of these variants was as adaptive as the parent strain, yet it is conceivable that variation which is nonadaptive in the nutrient broth environment might be adaptive in some different environment. Essentially this possibility was observed in a comparison of four different strains grown together in several environments. One strain completely replaced all others when grown at high temperatures, yet was markedly less adaptive than the other strains in all other environments tested. Had such a mutation occurred in a culture growing in this particular environment, it could be expected that this mutant eventually would have become the predominant type; occurring in any of the other environments this same mutation would have been lost because other types were more adaptive.

In higher organisms most mutations are deleterious or nonadaptive. If most bacterial variation is nonadaptive, it is expected that the greatest amount of variation will be found shortly before maximum growth of the population has occurred on a given media and environment. In any environment the least adapted genotypes are lost or occur at a low frequency, whereas those genotypes more adapted increase in frequency. After environmental changes, formerly suppressed types of mutants that arise during growth may replace types adapted in the earlier environment. Stock cultures that have been grown on a certain medium for a long time would not be expected to show sudden changes in their distinguishing characteristics as frequently as freshly isolated cultures or as old

stocks grown under different environments, because selection for a type adapted to grow on the stock media would already have occurred, whereas selection after change to a new environment may cause rather wide shifts in characteristics before stabilization takes place.

When a culture is observed at intervals over a period of time, there may be a gradual transformation of one cultural character into another. The concept of a gradual change is one that is common in bacteriological literature but one that often is interpreted as a phenomena in which all or most of the individual cells making up the culture change together in a definite direction. In the work discussed above evidence has been presented to show that variation originates as mutation of normal cells, at a rate probably characteristic for each strain of bacteria in any specific environment, and selection of types better adapted to that environment may then take place. The gradual change in the characteristics of a culture then becomes one of changing the frequency of occurrence of the individual cells of each specific genotype, the aggregate of which makes up a culture.

There is much evidence to support the view that evolution in bacteria is controlled by forces similar to those known to affect evolution of the higher organisms. In this paper evidence has been given that mutation and selection are important forces in changing bacterial populations, mutation being the source of genetic variation upon which selective forces may be effective. After variation is provided, evolution may proceed subject to selective forces. Under this view the static nature of a population implied by the term "pure culture" is misleading and highly problematic a few generations after a single cell is isolated.

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#### SUMMARY

The mutation rate of two stocks of *Phytomonas stewartii* was determined at five growth temperatures, 12, 18, 24, 30, and 36 C. Mutations were observed in colony color, surface appearance, and size. The rate of mutation at 36 C was approximately 10 times greater than the rate at 12 C, with intermediate mutation rates being observed at intermediate growth temperatures. The two stocks, similar in appearance except for colony color, one being dark yellow and the other pale yellow, differed significantly in their characteristic mutation rates. The kind and pattern of mutation were similar in the two stocks and at the test temperatures. The temperature coefficient ( $Q_{10}$ ) of the dark yellow stock was about 5.0 and of the pale yellow stock approximately 2.5. Certain characters became very mutable at growth temperatures of 30 to 36 C.

The mutation rate of six stable mutants derived from the dark yellow stock

400 when grown at 24 C in nutrient broth varied from about  $2 \times 10^{-7}$  to  $400 \times 10^{-7}$ , with the rate for the parental stock being about  $180 \times 10^{-7}$ .

The formula  $t = -\frac{1}{u} \ln \frac{q_1}{q_0}$  was derived to show the generations necessary for a given mutant type to increase to a given proportion if the change in types were due to mutation alone. At the highest mutation rate observed in these stocks 250 generations are needed for mutation alone to effect a 1 per cent increase in a mutant type.

Selection as a force in changing frequency of occurrence of a given type in a bacterial population was studied with mixtures of two or more morphologically distinct stocks. By starting with known proportions of each type the change in the proportion of these types could be followed during growth by plating at the desired time intervals. Rapid shifts in the occurrence of types were observed indicating that selection may be a strong force in changing bacterial populations.

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# THE EFFECT OF PODOPHYLLIN ON *EBERTHELLA* TYPHOSA

THOMAS H. GRAINGER, JR.

*Department of Biology, Lehigh University, Bethlehem, Pennsylvania*

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Recently, podophyllin, which is a resin of "podophyllum" and similar in many respects to colchicine, has been used in cytological research. Sullivan and Wechsler (1947) report the similarity of the action of podophyllin to that of colchicine on young, growing root tips of *Allium cepa*. The spindle mechanism was evidently impaired, and pronounced cytological effects were noted in the late prophase. The present report is concerned with the effect of podophyllin on two strains of *Eberthella typhosa*.

It was of interest to note whether there would be any changes in the colonial character of two strains of *Eberthella typhosa* when exposed to a saturated solution of podophyllin in nutrient broth.

The one strain of *Eberthella typhosa* used in this study produced typical S type colonies on nutrient agar. A single cell of this culture was isolated and put into nutrient broth for a previous study concerning the effects of X-rays on this strain (Grainger, 1947), as well as for this study. The strain was characteristic of the species in respect to all the biochemical and physiological characteristics as described in *Bergey's Manual* (1939).

The other strain of *Eberthella typhosa* used produced typical R type colonies on nutrient agar. It had been isolated recently in another study and was characteristic of the species in respect to all the biochemical and physiological characteristics as described in *Bergey's Manual* (1939), except for one difference—this strain would not ferment the sugar galactose.

The resin of podophyllum (Merck) used in this study was found to be only slightly soluble in water and gave a light brown color to the solution. A small amount (1 gram) was added to each of two flasks that contained 100 ml of nutrient broth. This amount allowed for a well-saturated solution of podophyllin in the broth. The reaction was adjusted to pH 7.0, and the material was then sterilized.

One loopful of a 24-hour nutrient broth culture of the S strain of *Eberthella typhosa* was placed in the flask of nutrient broth containing the podophyllin. One loopful of the same S strain was also added to a flask containing 100 ml of nutrient broth. This served as a control. The same procedure was followed with the R strain of *Eberthella typhosa*. The flasks were then placed in the incubator at 37 C.

Subcultures were made daily on nutrient agar plates by the streak method from the flasks containing the S and R cultures with the podophyllin in the nutrient broth, as well as from the flasks of nutrient broth which served as the controls. The colonies were studied by means of a colony microscope lens (3X) to note any changes in morphology. At least 100 well-isolated colonies were

studied daily on the nutrient agar obtained from the subcultures from each flask for a period of 30 days. There was no difference noted in the colonies from either the S or the R cultures of *Eberthella typhosa* in the flasks containing the podophyllin in nutrient broth, as compared with the S and the R colonies from the control flasks of nutrient broth. Occasionally, however, an intermediate form was observed from the S culture from the flask containing the podophyllin, but this was also observed from the control broth. There was no difference in the colonial character noted from the R culture in either the test flask or the control.

#### SUMMARY

The resin of "podophyllum" (podophyllin) saturated in nutrient broth did not have any effect on the colonial character of a S or R strain of *Eberthella typhosa*.

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# ACTIVITIES OF TWENTY-TWO ANTIBACTERIAL SUBSTANCES AGAINST NINE SPECIES OF BACTERIA

FREDERICK KAVANAGH

*The New York Botanical Garden, New York 58, New York*

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Each addition to the ever-lengthening list of antibiotic substances increases considerably the difficulty of identifying them short of isolation in pure form. Methods of identification applicable to extracts, culture filtrates, and concentrates are needed because the substances are first obtained in these forms; isolation of the pure substance may require long and tedious work with relatively large amounts of material.

During the work in this laboratory, which led to the isolation and characterization of about ten new antibacterial substances, it was necessary to devise a procedure which would enable us to decide whether or not we were working with new substances.

The data in the literature appeared ample to enable us to identify an unknown substance by measuring its activity against several bacteria and comparing the activities with those reported for the previously described antibiotic substances. Frequently, however, several strikingly different activities against the same species of bacteria were reported for the same substance. When one value was ten times another, it was impossible to decide which was the correct one. Since the details of the test method, composition of the test media, concentration of the bacteria, strain of bacteria, temperature, and duration of incubation all influence, to varying degrees, the activities found for a substance, these inconsistencies could be explained. Some authors, however, gave either no information or incomplete information about their test methods. To attempt to identify an unknown with one of the known antibacterial substances, one might try to use the procedures of each author—an impossible task—or to obtain as many as possible of the known substances and to use them to compile a consistent body of data using one technique with a few selected strains of bacteria, as has been done here. No attempt has been made to compare the results obtained here with those in the literature because of the differences in technique, strains of bacteria used, composition of test media, and temperatures of incubation. The activities of many of the antibacterial substances that have been isolated from green plants and fungi are collected in a recent review (Kavanagh, 1947b).

## MATERIALS AND METHODS

This paper reports the activity of 17 naturally occurring and 5 synthetic antibacterial substances against 9 kinds of bacteria, as determined by serial dilution methods. Details of the antibacterial methods are given elsewhere (Kavanagh, 1947). Frequently more than one active substance occurs in the culture filtrate or concentrate; usually such a mixture must be resolved into

its components before they can be identified. The active material in a solution suspected of containing a new antibacterial substance was fractionated into acidic, neutral, and basic groups before an attempt was made to identify them. Such simple chemical tests as color reaction with ferric chloride, thermal and pH stability, and susceptibility to inactivation by clarase or penicillinase were made. The antibacterial activities of the fractions were then measured against the 9 species of bacteria. The activities against the other bacteria relative to that against *Staphylococcus aureus* were computed and compared with the relative activities obtained for the 17 naturally occurring antibiotic substances. The antiluminescent activities (Kavanagh 1947a) were also determined.

The values in table 1 are those obtained in the majority of tests, although a single measurement is unlikely to be less than one-half as great or more than twice as great. The reproducibility of the values is a property not only of the antibacterial substance but also of the species of bacteria. The measured activities are sufficiently consistent for purposes of identification, since no single measurement determines the identity of a substance.

*Bacteria.* The bacteria used were *Bacillus mycoides* ATCC 9634, *Bacillus subtilis* ATCC 9633, *Escherichia coli* ATCC 9637, *Klebsiella pneumoniae* 9997, *Mycobacterium phlei* ATCC 10142, *Mycobacterium smegmatis* (smegma) ATCC 10143, *Pseudomonas aeruginosa* ATCC 10145, the Heatley strain of *Staphylococcus aureus* ATCC 9144, and *Photobacterium fischeri*, the Doudoroff strain, obtained from G. Rake.

The *B. mycoides*, *B. subtilis*, and *E. coli* were "standard tester strains" of S. A. Waksman.

#### SUBSTANCES AND SOURCES

The antibacterial substances were obtained from the following: aspergillie acid from G. Rake of The Squibb Institute for Medical Research; citrinin from J. H. Bailey of the Winthrop Chemical Company; 4,6-dimethoxy-toluquinone from Harold Raistrick; dihydrostreptomycin trihydrochloride (M 2216-H2, about 740  $\mu\text{g}/\text{mg}$ ) from O. Wintersteiner of the Squibb Institute; gliotoxin from J. D. Dutcher of the Squibb Institute; helvolic acid (fumigacin) from E. A. Doisy of St. Louis University; "Hogeboom and Craig No. 1" from L. C. Craig of the Rockefeller Institute for Medical Research; kojic acid from the Commercial Solvents Corporation; mycophenolic acid from Harry Sobotka; patulin, penicillic acid, and spinulosin from Harold Raistrick; the crystalline salts of the penicillins from the Commercial Solvents Corporation; streptomycin trihydrochloride (M 2213, 840  $\mu\text{g}/\text{mg}$ ) from O. Wintersteiner; streptomycin trihydrochloride-calcium chloride double salt (109X28C, 715  $\mu\text{g}/\text{mg}$ ) and hydrogenated streptomycin trihydrochloride (144X390I, 800  $\mu\text{g}/\text{mg}$ ) from the Research Laboratories of Parke, Davis and Company; and streptothricin (425 units/mg) from R. T. Major of Merck and Company. The "Hogeboom and Craig No. 1" (Hogeboom and Craig, 1946) was thought by Doering, Dubos, Noyce, and Dreyfus (1946) to be identical with their "ustin." The activities of the dihydrostreptomycin and of the hydrogenated streptomycin were identical



when tested by the methods used here and are given in the table under dihydrostreptomycin. The 2-methyl-1,4-naphthoquinone was a commercial product and was used as received. The tolu-*p*-quinone was an Eastman product that had been recrystallized. Biformin (Robbins, Kavanagh, and Hervey, 1947*b*), pleurotin (Robbins, Kavanagh, and Hervey, 1947*a*), and cassic acid (Robbins, Kavanagh, and Thayer, 1947) were isolated in this laboratory. The biformin was the purest obtained; the other two substances were crystalline.

All the antibacterial substances were assumed to be pure unless there was a statement to the contrary. Except for the streptothricin the amount of impurities in the compounds was too small to affect the activities as measured by the serial-dilution method used here.

TABLE 1

*Minimum inhibitory concentration of antibacterial substances in micrograms per milliliter*

ANTIBACTERIAL SUBSTANCE	B. MY- COIDES	B. SUB- TILIS	S. AU- REUS	E. COLI	K. PNEU- MONIAE	P. FIS- CHERI	P. AERU- GINOSA	M. PHEI	M. SNEGMA
Aspergillie acid.....	2	4	4	62	13	1	1,000	125	16
Biformin.....	13	0.04	0.3	1.7	1.7	0.02	53	0.6	3.3
Cassic acid.....	4	8	8	1,000	500	0.25	>250	8	30
Citrinin.....	32	16	16	>1,000	—	16	—	125	250
Dihydrostreptomycin.....	0.25	0.5	0.05	0.25	0.15	200	4	0.25	1
4,6-Dimethoxy-toluquinone.....	32	4	1	250	125	2	1,000	32	16
Gliotoxin.....	0.25	0.25	0.15	25	6	0.25	500	4	4
Helvolic acid.....	4	16	1	>1,000	4	—	—	>32	>32
Hogeboom and Craig No. 1.....	1.6	0.8	6	>50	>50	1.6	—	6	13
Hydrogen peroxide.....	31	4	8	10	5	5	8	31	4
Kojic acid.....	2,500	620	1,250	2,500	620	2,500	5,000	2,500	310
2-Methyl-1,4-naphthoquinone.....	12	3	1.7	220	28	3	>400	14	36
Mycophenolic acid.....	500	250	250	500	>1,000	125	>1,000	500	250
Patulin.....	16	4	8	8	8	0.25	125	16	1
Penicillie acid.....	32	8	16	64	64	1	1,000	64	32
Penicillin G.....	30	0.03	0.016	14	110	16	500	14	450
Penicillin X.....	30	0.06	0.03	14	240	8	500	29	470
Pleurotin.....	3	0.2	0.8	>500	>500	6	—	>32	32
Spinulosin.....	125	125	63	250	250	>16	500	250	500
Streptomycin.....	0.13	0.25	0.03	0.25	0.13	200	4	0.25	1
Streptothricin.....	100	0.8	0.1	0.3	0.1	20	2	7	14
Tolu- <i>p</i> -quinone.....	4	1	1	25	13	0.06	125	16	4

## RESULTS

The values given in table 1 are the minimum concentrations of the substances in micrograms per milliliter that prevented evident growth of the bacteria for 24 hours at a temperature appropriate for each species of bacterium. The values for streptothricin are for the compound as received; correction for impurities was not made. The values for streptomycin and dihydrostreptomycin are computed for the free base. The two samples of streptomycin were equally active, as were the two samples of dihydrostreptomycin.

The three species of bacteria generally most sensitive were *B. subtilis*, *S. aureus*, and *P. fischeri*, a gram-negative bacterium. The absolute activities against *S. aureus* ranged from 0.016 for penicillin G to 1,250 for kojic acid, with most of the values less than 16 micrograms per milliliter. *B. subtilis* and *S.*

*aureus* were about equally sensitive. *P. fischeri* was the most sensitive organism for six of the antibacterial substances but was the least sensitive to streptomycin and dihydrostreptomycin. The least sensitive of the bacteria was *P. aeruginosa*, which was inhibited appreciably only by hydrogen peroxide, streptomycin, dihydrostreptomycin, and streptothricin. The data presented here, as well as those in the antibiotics literature, indicate that the activity of a substance against one species cannot be predicted from the knowledge of the activity against another species of bacteria.

The antibacterial substances can be put into two groups: those active against the gram-negative bacteria and those much more active against the gram-positive than against the gram-negative bacteria. The EC/SA ratio computed by dividing the minimum concentration that inhibits *E. coli* by that needed to inhibit *S. aureus* provides an index for placing the substance in one of two quite distinct groups. The naturally occurring antibacterial substances for which  $EC/SA \leq 16$  seems to include those generally recognized to be active against the gram-negative bacteria. The naturally occurring substances, for which data are reported here, that are not in the group active against *E. coli* have  $EC/SA > 100$ . The members of a group can be separated from each other by utilizing the activities against some of the other test bacteria.

The activities of the antibacterial substances have been reported as concentrations measured in micrograms per milliliter. In comparing compounds with the greatly different molecular weights found among these substances (from 34 for hydrogen peroxide to 581 for streptomycin base), molar concentrations and not weight concentrations should be compared. Compounds with equally active molecules but different molecular weights will then have the same activities, whereas a comparison on a weight concentration basis would indicate that the substance with the lower molecular weight is the more active. For example, penicillin G and streptomycin are equally active against *S. aureus* when molecular concentrations are compared. Many other such comparisons are possible using the data in table 1.

#### APPLICATION OF THE METHOD TO IDENTIFICATION OF AN UNKNOWN SUBSTANCE

As an example of the usefulness of the data of table 1 in identifying an antibacterial substance, results obtained with the crude culture filtrate from *Penicillium claviforme* may be cited. This filtrate was presumed to contain patulin (Chain, Florey, and Jennings, 1942, 1944). Since the concentration of the active substance<sup>1</sup> was unknown, the antibacterial activities against four bacteria relative to its activity against *S. aureus* were computed and are given in table 2.

The EC/SA ratio of two, being less than 16, put the unknown substance in the group active against *E. coli*. The naturally occurring members of this group include aspergillic acid, biformin, patulin, penicillic acid, spinulosin, streptomycin, and streptothricin. The high relative activity against *B. mycoides* or *B. subtilis* eliminated biformin, streptomycin, and streptothricin from consideration. The relative activities against *E. coli* and *K. pneumoniae* indicated

<sup>1</sup> Only one substance with measurable activity is assumed to be present.

that the substance in the filtrate was not aspergillie acid, penicillie acid, or spinulosin. Thus, only patulin remained.

The great similarity between the relative activities of patulin and penicillie acid (identical for two bacteria) emphasizes the necessity for considering the activity against all nine bacteria when attempting to identify an unknown substance. Antibacterial methods can make identification highly probable, but only chemical methods can make it certain.

TABLE 2

*Comparison of a filtrate containing an unknown suspected of being patulin with seven known substances*

SUBSTANCE	ACTIVITY RELATIVE TO <i>S. aureus</i> = 1			
	<i>B. mycoides</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
Filtrate.....	1	0.5	2	1
Aspergillie acid.....	0.5	1	16	3
Biformin.....	40	0.1	5	5
Patulin.....	2	0.5	1	1
Penicillie acid.....	2	0.5	4	4
Spinulosin.....	2	2	4	4
Streptomycin.....	4	8	8	4
Streptothricin.....	500	1	1	1

## DISCUSSION

Since specific chemical tests suitable for application to very dilute solutions or very impure preparations of antibacterial substances are unknown, use must be made of biological methods. The most useful biological property, the one that by definition is common to this chemically heterogeneous group of organic compounds, is the ability to prevent the growth of microorganisms. Detection of the presence of an antibiotic substance by means of its inhibition of a microorganism is relatively simple because only one organism is required. The use of antibiotic action to identify a substance, however, necessitates the use of several microorganisms and requires a knowledge of the action of all of the known substances against this same set of microorganisms. Furthermore, a standardized technique must be used with the unknown and with all of the identified substances.

Too much of the published data on the antibacterial activities are good only for putting a substance into one of two groups, those active against gram-positive bacteria or those active against both gram-negative and gram-positive bacteria (the substances with activity only against fungi are not considered here). Most of the "bacterial spectra" were made with medically important bacteria in an effort to ascertain possible therapeutic applications of the substances. Frequently such lists of bacteria contain few that are available in many laboratory collections, few that are relatively nonpathogenic, and few that might be valuable in identifying antibiotic substances. All of the antibacterial substances of

natural origin have been tested for activity against only one species of bacteria, *S. aureus*.

The nine species of bacteria used in this work were selected because they grew rapidly in simple media, were susceptible to some of the substances, and differed greatly in their susceptibility to different substances. This is the first use of a species of *Photobacterium* in an antibacterial test; the usual test with it is an antiluminescent one (Rake, McKee, and Jones, 1942; Kavanagh, 1947a). It proved to be one of the more valuable test bacteria. If these strains of bacteria are employed in the test procedures devised for them (Kavanagh, 1947), other workers should be able to obtain the activities given in table 1, thus eliminating the necessity of actually determining the activities of all of the known substances each time an unknown is identified.

If only one substance is to be identified, chemical purification and identification possibly would be less time-consuming than the antibacterial method.

#### ACKNOWLEDGMENT

I wish to express my thanks to the many firms and individuals whose generosity in providing the antibacterial substances made this work possible.

#### SUMMARY

The activities of 17 antibacterial substances of natural origin and 5 synthetic ones were measured against 5 gram-positive (2 acid-fast) and 4 gram-negative species of bacteria.

*Bacillus subtilis*, *Staphylococcus aureus*, and *Photobacterium fischeri* were the most sensitive, and *Pseudomonas aeruginosa* was the least sensitive, of the nine bacteria.

The tentative identification of patulin in a culture filtrate is given as an illustration of the application of the antibacterial method.

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# THE PRODUCTION OF MUTATIONS IN STAPHYLOCOCCUS AUREUS BY CHEMICAL TREATMENT OF THE SUBSTRATE

ORVILLE WYSS, WILSON S. STONE, AND J. BENNETT CLARK<sup>1</sup>

*Department of Bacteriology and the Genetics Laboratory, The University of  
Texas, Austin, Texas*

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The literature on the induction of mutations in microorganisms by irradiation might well begin with the report by Henri (1914) on the appearance of two new forms of the anthrax bacillus subsequent to exposing these organisms to ultra-violet light. That Henri appreciated the significance of his finding is evident from his conclusion, "La lumière apparaît donc ainsi comme un agent fondamental de l'évolution intervenant par l'attaque plus ou moins profonde des fonctions nutritives intimes de la cellule." However, since many of the implications of studies on bacterial mutations are apparent only by liberal use of analogy to the genetics of higher forms, similar studies were necessarily retarded awaiting the development of the modern concepts of gene mutations. The recent interest in bacterial genetics has resulted in excellent reviews on this subject (Luria, 1947; Braun, 1947). One observes that in addition to radiations a variety of other methods are available for the production of mutations. Auerbach (1945) demonstrated that mustard gas would produce mutations in *Drosophila melanogaster*, and a similar treatment of microorganisms with the nitrogen or sulfur mustards, acenaphthene, and other chemical agents has resulted in the enhancement of the mutation rate.

Many of the reports on mutations in microorganisms are concerned with selection of naturally occurring mutants from the population. For example, Pinner and Voldrich (1932) observed the production of occasional nonpigmented colonies of *Staphylococcus aureus*, when a strain of that culture developed from a single cell and grown in nutrient broth was streaked on nutrient agar. If the organisms were transferred routinely in nutrient broth containing 5 per cent pleural fluid with a high agglutinin titer for the *S. aureus*, the culture would finally appear to be almost a pure *Staphylococcus albus*. In some cases it is difficult to decide to what extent the factors of selection are operative. The mutations in aspergilli reported by Thom and Steinberg (1939) and Steinberg and Thom (1940) may involve selections, or they may be due entirely to induced mutation. By the addition of a wide variety of agents to the medium these authors consistently found mutations with aspergilli. Nitrite, in the acid medium used for molds, was particularly active in producing large numbers of

<sup>1</sup> This study was undertaken in co-operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions are not to be construed as necessarily reflecting the views or endorsement of the War Department.

mutant forms. These mutants were stable over a long series of transfers on normal media. The types of mutations observed were similar to those occurring spontaneously; the number of mutants, however, was increased tremendously by the addition of various substances to the medium. Most of the substances employed were selected because they reacted with the amino groups on proteins. The authors suggested that the chemicals reacted with the protein components of the genetic mechanism and showed that some reversion occurred when the mutants were grown in a medium containing excess *D*-lysine.

Using another approach, Stone, Wyss, and Haas (1947) increased the resistance of *S. aureus* to penicillin and streptomycin by irradiation of the substrate prior to inoculation. Several lines of evidence were presented in an attempt to rule out selection as the determining factor in these experiments. They suggest that modified substrate molecules may be assimilated by the organism and built into inexact replications of the genetic mechanism. To test this theory we have treated the substrate with a number of chemical agents and have measured the effect of such treatment on the mutation rate of *S. aureus*. In most

TABLE 1

*Increase in mutation to penicillin and streptomycin resistance by treatment of broth with hydrogen peroxide one hour prior to inoculation*

TREATMENT OF BROTH	TOTAL COUNT	PENICILLIN 0.05 UNITS/ML		STREPTOMYCIN 3 UNITS/ML	
		Colonies	Mutants/ million	Colonies	Mutants/ million
Control.....	470,000,000	2,300	4.9	8,800	18.7
6 ppm H <sub>2</sub> O <sub>2</sub> .....	330,000,000	50,000	151	230,000	696

cases unstable chemicals were used to treat the substrate so that at the time of inoculation no residuum remained to obscure the result by possible direct action upon the organism.

#### EXPERIMENTAL

The methods employed involved the detection of penicillin- and streptomycin-resistant mutants in *S. aureus*. They are essentially identical with those reported by Stone *et al.* (1947). The substrate, usually nutrient broth, was treated with the chemical agent and permitted to stand at room temperature for 1 hour. If tests indicated the disappearance of the agent, the medium was inoculated with about a million cells per ml from a young broth culture. Residual hydrogen peroxide was checked qualitatively by starch-iodine, by catalase, and by the titanium sulfate method of Bonet-Maury (1944). The latter method was employed with the Coleman spectrophotometer; it permitted quantitative measurements to 0.1 ppm, but in the presence of broth it was somewhat less sensitive. After incubation the assay for resistant mutants was made. The experiment reported in table 1 shows the plate counts when organisms are grown in untreated broth and in broth treated with 6 ppm hydrogen peroxide 1 hour before inoculation.

On agar containing 0.05 units of penicillin per ml 2,300 organisms out of the initial population of 470 million per ml of untreated broth were able to form colonies; of the organisms grown on the broth treated with hydrogen peroxide 50,000 out of a total population of 330 million per ml were able to grow. Thus the number of mutants per million was increased over 30 times. A similar situation exists with the streptomycin mutants. A number of colonies were picked from the plates made from the treated broth and the drug resistance was shown to persist even after serial transfer on plain nutrient agar. These organisms were no more resistant to peroxide, nor did they grow faster in the peroxide-treated broth, than the control organisms.

TABLE 2  
*Effect of concentration of hydrogen peroxide and other oxidizing agents  
on the mutation rate*

TREATMENT OF BROTH	MUTANTS PER MILLION	
	Penicillin	Streptomycin
	0.05 units/ml	3 units/ml
Control.....	8.4	26.7
H <sub>2</sub> O <sub>2</sub> 9 ppm.....	no growth	no growth
H <sub>2</sub> O <sub>2</sub> 3.....	400.0	1334.0
H <sub>2</sub> O <sub>2</sub> 1.....	26.7	84.0
H <sub>2</sub> O <sub>2</sub> .3.....	10.0	23.4
Cl <sub>2</sub> 125.....	no growth	no growth
Cl <sub>2</sub> 75.....	10.0	30.0
Cl <sub>2</sub> 6.....	12.0	23.0
I <sub>2</sub> 6.....	2.5	10.5
NaNO <sub>2</sub> 500.....	—	25.0
KMnO <sub>4</sub> 10.....	20.0	36.0
Control.....	8.0	33.4
Aerated with oxygen.....	7.0	30.0
Irradiated 15 minutes (ultraviolet light).....	180.0	700.0

Although at the time of inoculation no peroxide could be detected in the broth treated with 6 ppm H<sub>2</sub>O<sub>2</sub>, experiments on concentration were instituted to observe whether or not mutations would be induced by treatment of the broth with concentrations well below the amount inhibiting growth. As shown in table 2 the bacteria failed to make visible turbidity in 18 hours in broth treated with 9 ppm H<sub>2</sub>O<sub>2</sub>. However, even the treatment with 3 and 1 ppm peroxide resulted in a definite increase in the mutation rate.

The addition of 6 ppm Cl<sub>2</sub> or I<sub>2</sub> failed to produce any increase in the mutation rate. In fact, when the concentration of Cl<sub>2</sub> added to the broth was increased to a value just short of that which gave a free chlorine residual and thus prevented growth, the mutation rate to streptomycin and penicillin resistance still remained essentially that of the control. High concentrations of NaNO<sub>2</sub> failed to increase the mutation rate to streptomycin resistance (penicillin not tested), although in this case much of the nitrite remained in the broth at the time of inoculation. The nutrient broth used was of a neutral pH, so the reaction with

amino acids suggested by Steinberg and Thom could not be expected to occur in this experiment. Potassium permanganate added at a level which reacted completely with the broth failed to affect the mutation rate. Bubbling pure oxygen gas through the medium for 1 hour prior to inoculation did not have any effect on the mutation rate of organisms subsequently inoculated therein. These experiments suggest that the effect of hydrogen peroxide on the mutation rate is fairly specific and not merely the result of growing organisms in a medium with a high oxidation-reduction potential.

The time elapsing between the addition of 6 ppm of peroxide to the broth and inoculation was varied from 15 minutes to 22 hours without markedly affecting the result (table 3). Within experimental error the mutation rate was increased about 5- to 10-fold in the case of penicillin and 10- to 20-fold in the case of streptomycin. From these data it appears reasonable that the effect of the hydrogen peroxide is due to its reaction with some component in the medium.

TABLE 3  
*The effect of time elapsed between treatment of the broth with 6 ppm  
hydrogen peroxide and inoculation*

TIME BEFORE INOCULATION	MUTANTS PER MILLION	
	Penicillin	Streptomycin
	0.05 units/ml	3 units/ml
15 min.....	73.5	541.0
40 min.....	97.5	795.0
2 hours.....	74.8	621.0
3 hours.....	64.0	848.0
5 hours.....	60.3	578.0
22 hours.....	42.7	331.0
Control (no H <sub>2</sub> O <sub>2</sub> ) .....	9.4	46.4

In order to determine whether or not a selective action of the treated broth was responsible for the result the rate of appearance of the mutants in the young culture was studied. Platings made at 0, 3, 6, and 24 hours after inoculation indicated that in the peroxide-treated broth the mutants appeared at a rate that could best be explained by assuming that the mutations were induced by treated substrate. Very careful measurements on growth rates of mutant subcultures, of the parent strain, and of mixtures of the latter with mutant cultures indicate that in neither normal broth nor peroxide-treated broth did population changes occur which would permit attributing the results to a selective action.

Several chemical substances have been treated with hydrogen peroxide and then added to normal broth. For example, 100 mg of phenyl alanine were dissolved in 100 ml of water to which 100 ppm hydrogen peroxide were added. After an hour one ml of this mixture was added to 50 ml of broth and inoculated with *S. aureus*. After a suitable growth period the resistant mutants were determined and compared with results obtained with control cultures. The results show a considerable enhancement of the mutation rate. Much of the



hydrogen peroxide had disappeared from the amino acid solution before the latter was added to the broth, so it appears unlikely that the effect was due to the residual peroxide acting on the broth components.

Other substances giving the increased mutation rate when treated with peroxide are tryptophane, tyrosine, adenine, uracil, and guanine. Tryptophane is reported to be converted to indole acetic acid by the action of hydrogen peroxide or ultraviolet light, but the addition of indole acetic acid to the medium had no effect on the mutation rate. A number of reducing agents such as thioglycolic acid, sodium sulfite, and sodium sulfide had no effect on the rate of mutation.

Stahmann and Stauffer (1947) treated fungous spores with methyl-bis-( $\beta$ -chloroethyl)-amine and obtained a high mutation rate measured by colonial variation. The concentrations used (0.01 M) killed a large percentage of the mold spores in 30 minutes and showed a pronounced increase, not only in the fraction of the survivors which were mutants, but in the total number of mutants in the smaller surviving population. We employed tris-( $\beta$ -chloroethyl)-amine, which at equivalent molar concentrations showed about the same killing rate with *S. aureus* cells as the methyl derivative used by Stahmann and Stauffer exhibited with the mold spores. When 90 per cent of the *S. aureus* cells were killed, the rate of occurrence of penicillin-resistant cells was found to have increased 20-fold. When this substance was added to the broth at several concentration levels and permitted to react for 4 hours before inoculation, it resulted in a pronounced increase in the number of penicillin-resistant and streptomycin-resistant cells in the resulting population. It is believed from lack of odor and inhibitory action of the broth at the time of inoculation that the mustard had completely hydrolyzed before the cells were added. The action here, also, appears to be one of mutation induced by action upon the substrate.

The correlation between the action of hydrogen peroxide and ultraviolet light is difficult to determine. Irradiation of water by ultraviolet under the conditions of our experiments produces a considerable amount of peroxide. Similar irradiation of the broth produces no detectable residual peroxide since it appears to react quickly with broth constituents. Experiments in which catalase was added to the broth during irradiation and after treatment with hydrogen peroxide gave conflicting results.

#### SUMMARY AND CONCLUSIONS

These data indicate that treatment of broth with ultraviolet light is not a unique indirect method of inducing mutations. Hydrogen peroxide reacts with some broth components, and an increase in mutant forms appears when organisms are grown in their treated medium, although no peroxide remains at the time of inoculation. A similar action occurs with a nitrogen mustard.

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# THE KAHN REACTION IN RABBITS IN RELATION TO THEIR AGE

STANLEY MARCUS<sup>1</sup> AND REUBEN L. KAHN

*Serology Laboratory, University Hospital, University of Michigan, Ann Arbor, Michigan*

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The relationship between age and immunity has long been recognized. The "diseases of childhood" and the Shick and Dick susceptibility curves in the young furnish illustrations of this relationship. Hirsfeld, (Halber *et al.*, 1927) proposed the theory that certain so-called "normal" antisubstances (e.g., isohemagglutinins) are inherited, and these become functionally mature at some specific time in the individual's development—a characteristic referred to as "serological maturity." Baumgartner (1934) has reviewed the literature on the relationship of age to immunological reactions through 1933 and has given evidence to substantiate Hirsfeld's hypothesis. Kahn (1936) demonstrated that the localizing power of the cutaneous tissues for protein antigens possessed by immune adult rabbits is much more marked than the similar capacity of young rabbits.

Wendt (1925) claimed that, though the majority of sera from cattle were Wassermann-positive (icebox fixation), calf sera were usually negative. Mackie and Watson (1926) observed that the sera of adult cattle, sheep, and rabbits, with few exceptions, gave positive reactions with the Wassermann (37 C fixation) or Sachs-Georgi tests, but the sera of some calves, lambs, and pooled young rabbits were usually negative. Sherwood, Bond, and Clark (1941) and Kemp, Fitzgerald, and Shepherd (1940) have reaffirmed these observations and reported similar findings with beef, dog, and sheep sera.

In the experiments to be described serologic studies with sera from rabbits were made in relation to the age of the animals. Newborn rabbits had their birthdays tattooed on their ears and were first tested serologically when they became about 45 to 55 days old. They were then retested at intervals of 14 to 20 days until five or more examinations had been made. The weight of the animals when first tested was about one pound, and the blood was obtained by cardiac puncture. The Kahn standard and differential temperature tests (Kahn, 1946) were employed, and they were performed on unheated portions of serum as well as on portions heated at 56 C for 30 minutes.

Thirty-one animals, including 10 females and 21 males, were used in the study. The results in nine representative cases are included in table 1. The tabulated results of the differential temperature tests include only the reactions obtained at 1 C. The reactions obtained at 37 C were found to be essentially negative and were not included in the table.

## RESULTS

It will be noted from the representative cases listed in table 1 that all animals gave negative flocculation reactions with the standard Kahn test when first

<sup>1</sup> Present address: Horace H. Rackham Arthritis Unit, University of Michigan Hospital.

TABLE 1  
Increase in potency of serologic reactions in rabbits with age

AGE	KAHN REACTION		DIFFERENTIAL TEMPERATURE REACTION (AT 1 C)	
	Heated serum	Unheated serum	Heated serum	Unheated serum

Rabbit 497 (female)				
Days				
52	-	-	-	-
64	-	+	-	+
82	-	+	-	+
104	+	+	+	+
131	-	+	+	+

Rabbit 481 (female)				
51	-	-	-	-
63	+	+	+	+
81	+	+	+	+
103	+	+	+	+
130	+	+	+	+
158	+	+	+	+
170	+	+	+	+

Rabbit 148 (female)				
56	-	-	-	-
68	-	+	+	+
86	-	+	+	+
108	+	+	+	+
135	+	+	+	+

Rabbit 482 (female)				
51	-	-	-	-
63	-	-	-	-
81	-	+	+	+
103	+	+	+	+
130	+	+	+	+



examined at ages of approximately 45 to 55 days. When unheated serum, instead of serum heated at 56 C, was employed, the same test gave negative results in all instances except one in which a very weak reaction was noted. When the tests were conducted at 1 C with heated serum, again only one rabbit gave a weak flocculation reaction; with unheated serum, however, two definite flocculation reactions were obtained. It would appear that the sera of rabbits under 2 months of age show little or no serologic reactivity with lipid antigen.

On successive examinations the Kahn standard test did not begin to give positive reactions until the rabbits were approximately 3 months of age. But Kahn tests with unheated sera began to give positive reactions at about 2 months of age. Reactions appeared earliest, remained most consistent, and reached their highest quantitative levels in the case of the unheated sera tested at 1 C.

Seven of the 31 rabbits tested showed reactions typified by rabbit 491, i.e., a persistently negative Kahn reaction in the standard test, weak fluctuating reactions with heated sera at 1 C, and fairly consistent flocculation with unheated sera both at room temperature and in the cold. The majority of animals showed the patterns illustrated by rabbits 148, 498, 487, and U493 in the table, beginning with negative reactions when under 2 months of age, approaching positivity between 2 and 3 months, and remaining positive after that time. A few of the animals showed patterns which varied somewhat from the above, illustrated by rabbits 497, 478, 481, and 482.

#### SUMMARY

Data are presented showing that Kahn reactions given by rabbits tend within limits to become stronger with the increase in age of the animals. Rabbits under 2 months of age are generally sero-negative. The appearance of positive reactions after 2 months is relatively common. Unheated sera tend to give stronger Kahn reactions than sera that have been previously heated at 56 C for 30 minutes. Highest quantitative titers are obtained when the tests are conducted at a low temperature (1 C) with unheated sera.

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## THE NATURE OF ACID-FASTNESS

DIRAN YEGIAN AND ROBERT J. VANDERLINDE

*Ray Brook State Tuberculosis Hospital, Ray Brook, New York*

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Many theories have been proposed to explain the acid-fast property of the tubercle bacillus, but no satisfactory explanation has yet been given. It is suggested that the property is dependent upon the permeability of the cytoplasmic membrane. Evidence will be presented in support of this concept. It will be shown that when the Ziehl-Neelsen technique is employed the dye exists within the cell in two distinct portions: a small portion is bound to the cytoplasm and the remainder is free. The characteristic color of the stained bacillus is due to the free dye which can be removed without altering the acid-fast property. In the past, investigators (Koch, 1897; Aronson, 1898, 1910; Tamura, 1913; Anderson, 1932) have believed that this phenomenon of acid-fastness might be based upon the properties of chemicals isolated from the tubercle bacillus or of complexes of these chemicals. At one time there was a general belief that the presence of a wax sheath around the cell was responsible for its peculiar staining characteristics, but this is no longer tenable after the study made by Knaysi (1929). Some authors including Ehrlich thought that acid-fastness was related to the permeability of the cell membrane, but this structure was not clearly defined and experimental evidence was not given. The one point on which there is complete agreement is that the integrity of the cellular structure must be maintained to preserve the acid-fast property.

Since our proposed explanation of the mechanism of acid-fastness is based on the function of a cellular structure, a brief review of pertinent information on the cytology of the tubercle bacillus is indicated.

The cell wall of the tubercle bacillus has been observed directly with the electron microscope by Mudd and Anderson (1944). Mudd and Mudd (1927) demonstrated the hydrophobic property of the surface of the bacillus. The apparent functions of the cell wall are to protect the cell from mechanical injury and to impart to the cell its characteristic shape.

The electron microscopic studies of Mudd, Polevitsky, and Anderson (1942) and Mudd and Anderson (1944) give direct evidence of the cytoplasmic membrane in bacteria. According to Knaysi<sup>1</sup> (1929, 1938, 1944, 1946), who refers frequently to this structure, the membrane of the tubercle bacillus probably consists of lipids and protein. With ordinary technique it appears to be the external boundary of the cytoplasm rather than a separate structure.

### EXPERIMENTAL RESULTS

Although there is some evidence that the cell wall of the tubercle bacillus retains small amounts of certain other dyes, our observations suggest that this

<sup>1</sup> A recent personal communication from Knaysi states that what he referred to as the cell "membrane" in his paper (1929) is now usually known as the cytoplasmic membrane.

structure does not stain acid-fast to a degree that would add to the color of the bacillus as seen by the ordinary light microscope. The following observation indicates that the portion of the cell which is acid-fast-staining is within the cell wall.

Cells from a young, actively growing culture of tubercle bacilli are stained solidly by the Ziehl-Neelsen technique. Later, because of prolonged unfavorable environmental conditions, granular forms appear. In these older bacilli the cytoplasm separates into granules that vary in size and number and stain strongly acid-fast. Ordinarily no stained material is seen between the granules, but occasionally, when separation of the cytoplasm is incomplete, a faintly stained line may be seen connecting two granules. We believe that when the separation of the cytoplasm is complete the cytoplasmic membrane surrounds the individual granules and is no longer continuous over all the granules in a single bacillus. When a granular cell is stained first by the Ziehl-Neelsen method and then outlined by nigrosine, a white continuous border is seen surrounding the acid-fast cell. This represents the unstained cell wall which retains the granules and prevents the nigrosine from outlining the individual granules (figure 1).

It is our belief that when the Ziehl-Neelsen technique is employed the dye gains entrance into the interior of the cell through the cytoplasmic membrane, where a small portion of the dye is firmly absorbed by the cytoplasm and can be removed from the cell only with difficulty. The remaining free dye is held in the cell because it is unable to diffuse through the cytoplasmic membrane, and it is to this free dye that the characteristic red color of the stained bacillus is due.

If the greater portion of the dye in the cell can be shown to act as though it can be precipitated and then redissolved, it is evidence that the dye behaves as free dye. When a film of tubercle bacilli, stained with either the acetate or hydrochloride of rosanilin to which 0.5 per cent of sodium chloride is added, is examined microscopically, the dye appears to be evenly distributed throughout the cells and of uniform density (figure 2). The free dye in these stained bacilli can be precipitated and redissolved by the following process, which may be observed microscopically.

The application of acid alcohol to the film causes the color of the bacilli to change to a bluish red. If the film is now washed with water, there will be noted a sudden shift of the dye to certain points in the cell, resulting in the formation of one or more red-black bodies (figure 3). These bodies, which are referred to as beads, are often considerably greater in diameter than the width of the cell. The areas between the beads stain faintly, and the width of the cell in these areas is diminished. These faintly stained portions contain the dye that is absorbed by the cytoplasm.

The accumulated or precipitated dye which forms the beads can be dissolved almost instantaneously by the application of either 5 per cent phenol or 95 per cent ethyl alcohol, following which the dye spreads evenly throughout the cell and gives the cell a uniformly bright red appearance (figure 4). The process of accumulation and redistribution of the dye may be repeated in the same cell without restaining, but eventually beads will fail to form. Apparently some



dye is removed in the course of the process, and a certain minimum amount of free dye is necessary for precipitation. Yegian and Baisden (1942) have shown that when the concentration of the dye is reduced the beads decrease in size and number but not in density.

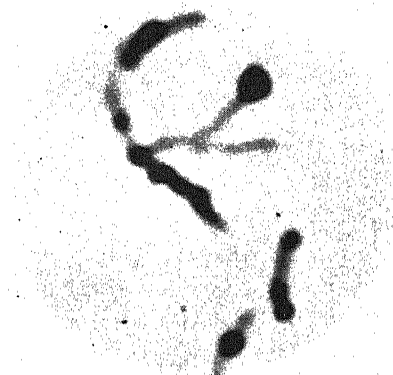
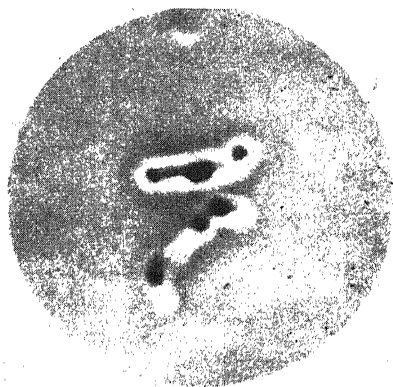


FIG. 1. Upper left: The granular form of the tubercle bacillus stained by the Ziehl-Neelsen technique, with its unstained wall outlined by nigrosine.

FIG. 2. Upper right: Tubercle bacilli stained with carbol fuchsin containing sodium chloride. The dye is evenly distributed throughout the microorganism.

FIG. 3. Lower left: The same cells as in Figure 2, washed with acid alcohol and then with water. The dye has been precipitated to certain points in the cell.

FIG. 4. Lower right: The same cells as in Figure 3, washed with 95 per cent ethyl alcohol. The dye is again evenly distributed throughout the cell.

All illustrations were taken at a magnification of 1,200, then enlarged three times.

Further observations support our contention that the formation of beads represents an accumulation or precipitation of free dye within the tubercle bacillus.

(1) The location of beads cannot be predicted before formation, nor can evidence of their previous location be found after dispersal with alcohol or phenol. When reformed, beads may be found in their original location, but just as often they occupy new positions in the cell (Porter and Yegian, 1945). These findings as well as the fact that the beads may be produced in cells from which the ether-

alcohol-soluble constituents have been removed, make it very unlikely that beads are formed about a pre-existing structure within a fixed organism.

(2) The rate at which the precipitated dye is dissolved does not resemble the gradual withdrawal of dye from a stained structure. The addition of an electrolyte, such as sodium chloride, to a solution of carbol fuchsin results in the formation of a precipitate which, when separated by centrifugation, is readily soluble in alcohol.

(3) The process of bead formation is almost instantaneous. Occasionally we have noted that beads were not formed so soon as water reached the preparation but that a slight tap on the bench supporting the microscope coincided with sudden bead formation. The effect of vibration on precipitation is well known.

(4) The time necessary to cause beads to disappear is longer when stained films are kept for several weeks and then treated with alcohol. This suggests that the beads in older films contain less liquid.

(5) The granular form of the tubercle bacillus may be stained to show beading, but it will be noted that the dye accumulates and is dispersed only in the granular portions and not in the unstained interspaces. This finding is further support for the contention that in these granular cells the cytoplasmic membrane has separated and is about the individual granules which are retained in the cell by the cell wall.

(6) The phenomenon of accumulation or precipitation of free dye may be observed within cells other than mycobacteria. Certain mushroom spores when stained resist decolorization. They may also show bead formation and subsequent dye dispersal when stained and treated by the method previously described.

Our original hypothesis would be further supported if we could show that the free dye can be removed from the cell without altering its acid-fast property.

The following method was employed to demonstrate the removal of free dye. Two suspensions of tubercle bacilli, strain H37, were made: one in distilled water, and one in which the bacilli were mixed with an approximately equal amount of "tween 80" (Dubos and Davis, 1946) previous to suspension in distilled water. Both suspensions were boiled in a water bath for 20 minutes, and a loopful of each was placed on a slide. The films were dried in air and then stained by the usual Ziehl-Neelsen technique (Yegian and Budd, 1943).

All visible dye was removed from the films after immersion in either boiling neutral 50 per cent alcohol or boiling water. It was observed that the dye was removed from the films containing "tween" in 30 to 60 seconds as compared with 5 to 7 minutes for those which did not contain "tween." The dye from both films was removed more rapidly by immersion in boiling alcohol than by immersion in boiling water.

When the films from which all free dye had been removed were examined microscopically, the individual bacilli appeared faintly pink. To remove this last faint trace of dye absorbed by the cytoplasm required considerably longer immersion. The change in staining that occurred as the result of the initial immersion was so marked that one would not hesitate to say that the cells had

lost the coloring that is usually the basis for calling bacteria acid-fast. When restained by the Ziehl-Neelsen method, the bacilli in both preparations were acid-fast. When the cells are counterstained with brilliant green following the immersion procedure, they appear brilliantly stained, showing that the free dye which was removed was probably the source of the acid-fast coloring.

When films of bacteria from which the free lipid had been extracted were prepared and stained as before and then immersed in boiling water or alcohol, the time required to remove the dye was not altered.

We believe that the decolorization procedures described here have their basis in the alteration of the permeability of the cytoplasmic membrane by the boiling solutions, but that the change is not permanent is evident when the Ziehl-Neelsen procedure is repeated and the bacilli are found to be acid-fast. The action of "tween 80" is not understood, but it may, by altering the permeability of the cytoplasmic membrane, permit the more rapid removal of free dye.

#### DISCUSSION AND SUMMARY

The foregoing observations lead us to propose an explanation for the acid-fast characteristic of mycobacteria that is both simple and comprehensive. Our hypothesis is supported by the fact that, during the staining procedure when the Ziehl-Neelsen technique is employed, fuchsin enters the cell through the cytoplasmic membrane and is not removed by the acid alcohol used in the procedure. Fuchsin exists within the cell in two forms: (1) One is a form which can be accumulated in beads and acts as free dye. It is to this form that the usual color of the stained acid-fast bacillus is attributed. (2) A small portion of the dye acts as though firmly bound by the cytoplasm and gives the organism only a very faint pink color.

Lamanna (1946) has postulated that beading is the result of phenol and dye separating out as a liquid phase. We have been able to show that mycobacteria will exhibit acid-fastness and beading even after extraction of the free lipids. Since we have not noted any progressive decrease in intensity of acid-fast coloring during the stages of extraction, we do not believe that the acid-fast property lies in the greater solubility of phenol and dye in the cellular lipids than in the decolorizing agent.

Anderson (1932) isolated mycolic acid and has brought evidence to show that mycolic acid exists as a lipopolysaccharide in the cell and that this complex is acid-fast. Long (1922) believed that bound lipid existed as a lipoprotein in the cell and that this complex determined cell permeability. Boissevain (1927) believed that acid caused hydrolysis of an acid-fast substance in the cell and thus loss of acid-fastness.

In our experience and in that of Rich (1944) mycolic acid and the other extractable lipids are not strongly acid-fast, and when extremely thin films of mycolic acid are stained they appear only faintly acid-fast. Also, no mycolic acid or closely related substance has been extracted from certain acid-fast structures other than mycobacteria. The fact that lipids extracted from tubercle bacilli are only faintly acid-fast and that without drastic procedures, such as the

use of acid in the extraction, the bacilli remain acid-fast suggests that the relation of extractable lipids to acid-fastness has been overestimated. Although it is known that acid hydrolysis will split lipoprotein complexes in certain cells, it is also known that acid can affect a variety of other cellular components and structures, some of which may be important in the property of acid-fastness. Extraction of a chemical from a cell may upset the function of a structure in the cell in some demonstrable way, but it does not necessarily follow that the extracted chemical or one of its complexes within the cell represents the major factor in the function of the structure. It may be true that certain lipo-complexes are controlling factors in the permeability property of the cell, but in itself the permeability function of the intact cytoplasmic membrane needs emphasis.

We have shown that apparently only a small portion of the dye is bound to cytoplasm. The essential characteristics of the beading process, such as the visible shift of the dye within the cell, the variability of location of the beads in the same bacillus after dissolution and reformation, and the failure to demonstrate any structure about which the beads might be formed all substantiate the fact that the major portion of the dye behaves as would be expected of a free dye. The process of beading resembles precipitation very closely, and evidence has been offered for this idea.

We have shown that the free dye, which gives the usual color to the stained bacillus, can be removed by immersion in boiling alcohol or water without altering permanently the acid-fast property.

The fact that tubercle bacilli are rendered non-acid-fast by trauma, autolysis, or the use of acid is satisfied by our theory; the action has been so severe that the cytoplasmic membrane no longer controls permeability with relation to the dye used.

The appearance of acid-fast mushroom spores stained by the Ziehl-Neelsen procedure to show beading and the reaction of the resulting beads to phenol or alcohol give us some evidence that the existence of free dye that can be accumulated and then redistributed within a structure is not peculiar to mycobacteria. Certain acid-fast structures other than tubercle bacilli have been shown to contain only small quantities of lipids. The theory we are proposing can account for the acid-fastness of these structures as well as of the mycobacteria.

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# ANTIBIOTIC INTERRELATIONSHIPS AMONG THE ENTERIC GROUP OF BACTERIA

PIERRE FREDERICQ<sup>1</sup> AND MAX LEVINE

*Iowa State College, Ames, Iowa*

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Instances of antagonism among the enteric group of bacteria have been frequently reported in the literature, but these are concerned primarily with mixed cultures in which many different factors, difficult to analyze, are involved. In 1925 Gratia observed a strain of *Escherichia coli* which produced a diffusible and thermolabile antibiotic substance. He called this substance "principe V." This "principe V" seemed to be specific and at the time appeared to be active against only one other strain of *Escherichia coli* and *Shigella dysenteriae*. Later, Gratia and Fredericq (1947) found that antibiotic strains of *E. coli* are not at all infrequent, but that the antibiotic spectrum of the different strains was characteristically different and distinct.

The antibiotic substances produced, to which was assigned the term "colicin" (Gratia and Fredericq, 1947), differ not only in their antibiotic spectra but also in their physiochemical properties (Gratia and Fredericq, 1946). It has been observed that some strains may produce several colicins with characteristically different antibiotic spectra. It was furthermore observed that practically every member of the *Enterobacteriaceae* studied, including the strains which themselves produced antibiotic colicins, was sensitive to one or more colicins produced by some other member of the *Enterobacteriaceae*.

In the present study of antibiotic interrelationships among members of the enteric group of bacteria we employed the following technique (Fredericq, 1946): A small (about 1-mm) loop of a broth culture of a strain to be tested for production of antibiotics was stabbed on previously poured and dried peptone agar plates. After incubation for 48 hours at 37 C the culture was killed by exposure to chloroform vapors for a period of about 1 hour, and the chloroform was then allowed to evaporate. The entire surface of this agar plate was then inoculated with a strain being observed for sensitivity. A convenient procedure for this purpose consisted of covering the surface of the medium with a sterile filter paper and then inoculating this filter paper with a ml or two of a culture of the organism being studied for sensitivity. After contact for several minutes the filter paper was removed and the plate incubated for 24 hours at 37 C. If an antibiotic was produced, growth of a sensitive strain was inhibited in a circular zone in the vicinity of the stab but not restricted on the rest of the plate, as may be seen in figure 1. It will be observed that two strains were markedly antibiotic, one only very slightly, and the fourth showed no evidence of antibiosis against a strain of *Escherichia* being tested for sensitivity.

The antagonistic action under consideration appears to be quite different

<sup>1</sup> Fellow of the Belgian-American Educational Foundation.

from the autoinhibition of coliform bacteria reported by Powers and Levine (1937) and Coblentz and Levine (1947) when employing the so-called "staling" technique, which consists of preparing agar by adding a 3 per cent agar gel to an equal volume of an old broth culture of the test organism and then observing growth after reinoculation of this medium. Thus, from table 1 it will be noted that *Escherichia* strain CA1 did not grow on a staled medium made with that same culture, but it was not autoinhibitory when employing the antibiotic

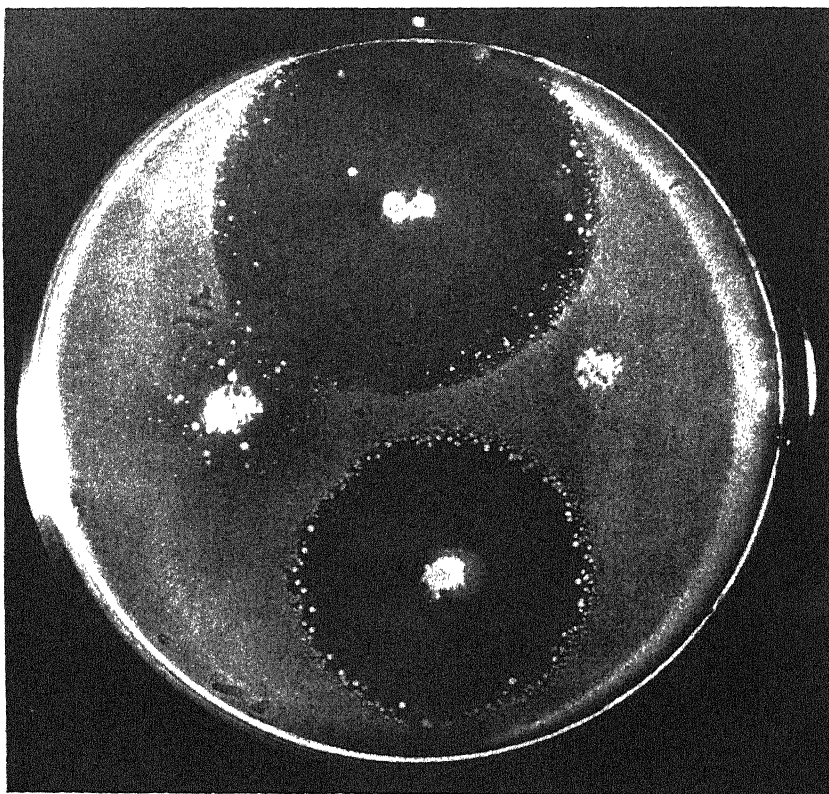


FIG. 1. ANTIBIOTIC ACTIVITY AMONG ENTERIC BACTERIA

technique described above. *Escherichia* strain CA2, *Shigella* CA44, and paracolon CA79 similarly show autoinhibition of growth by the staling technique but offer no evidence that a diffusible autoinhibitory substance is produced when they are tested by the antibiotic technique. On the other hand, strains CA1, CA2, and CA44 show no evidence of inhibition against strain A9, when the staling technique is employed, but marked inhibition when the antibiotic technique is employed. Strain CA44 is particularly striking in that it is antibiotic against a number of other strains but not, however, against itself. The two substances associated with growth inhibition—namely, that which is active



in the staling technique and that which is responsible for growth inhibition by the antibiotic technique—would, therefore, have to be considered as probably distinct until their nature is more definitely determined.

TABLE 1

*Growth inhibition on staled agar and in vicinity of colonies of antibiotic strains*

STRAIN OBSERVED FOR GROWTH INHIBITION		<i>Escherichia</i> CA1	<i>Escherichia</i> CA2	<i>Shigella</i> * CA44	Paracolon CA79	<i>Escherichia</i> CA8	<i>Escherichia</i> A9	<i>Citrobacter</i> CA33	<i>Aerobacter</i> A6
Antibiotic strains employed	Technique	Inhibition of growth							
<i>Escherichia</i> CA1	S	+	+	+	+	+	—	—	—
	A	—	—	—	—	—	+	—	—
<i>Escherichia</i> CA2	S	+	+	+	+	+	—	—	—
	A	—	—	—	—	—	+	—	—
<i>Shigella</i> * CA44	S	+	+	+	+	+	—	—	—
	A	+	+	—	+	—	+	+	+
Paracolon CA79	S	+	+	+	+	+	—	—	—
	A	—	+	—	—	—	—	+	—

A, antibiotic technique (2-day culture, 37 C).

S, staled medium prepared from 10-day culture, 37 C.

\* Final allocation not definitely made.

TABLE 2

*Frequency of antibiosis among strains of enteric bacteria*

STRAINS TESTED FOR GROWTH INHIBITION	GROUP...	<i>Escherichia</i>	Paracolon	<i>Citrobacter</i>	<i>Aerobacter</i>	<i>Proteus</i>	<i>Salmonella</i>	<i>Eberthella</i>	<i>Shigella</i>	All Groups
No.....		56	23	13	14	8	33	6	41	194
Antibiotic strains		Per cent of trials showing growth inhibition								
Group	No.									
<i>Escherichia</i>	39	37	20	10	2	4	22	19	44	28 (7,566)*
Paracolon	21	22	12	7	0	0	6	9	44	18 (4,074)
<i>Citrobacter</i>	8	26	20	19	0	0	7	25	47	23 (1,552)
<i>Eberthella</i>	5	15	8	17	0	0	0	17	11	10 (970)
<i>Shigella</i>	15	17	7	1	0	0	0	3	36	13 (2,895)
All groups	88	28	15	9	1	2	12	14	40	22 (17,057)
		(4,928)*	(2,024)	(1,144)	(1,232)	(704)	(2,904)	(528)	(3,593)	

\* Figures in ( ) indicate number of trials or observations (product of numbers of antibiotic and test strains).

Table 2 shows the frequency of the phenomenon of antibiosis among strains of enteric bacteria. Eighty-eight strains were tested for antibiotic potency against 194 strains of the colon group (the latter including the test strains). Of 17,057 observations, evidence of growth inhibition was observed in 22 per

cent. Considering the sensitivity of 56 strains of *Escherichia* to antibiotics produced by other enteric bacteria, it will be noted that growth inhibition was observed most frequently (37 per cent of the trials) when *Escherichia* strains were tested against other *Escherichia* strains and least frequently (17 per cent) when they were tested against *Shigella* strains.

The paracolon bacilli showed results similar to those observed for the *Escherichia*.

The *Citrobacter* strains were most frequently inhibited by other strains of *Citrobacter* or by *Eberthella* strains, but it should be noted that the number of antibiotic strains, 8 and 5, respectively, are too few for final judgment.

As a group, the *Shigella* strains seem particularly prone to be sensitive to antibiotic agents produced by enteric bacteria. Thus, growth inhibition was observed in 40 per cent of the trials with *Shigella* as compared to 28 per cent with *Escherichia* and 12 per cent with *Salmonella*.

In the 14 strains of *Aerobacter* and 8 strains of *Proteus* studied, sensitivity was only occasionally observed; and then only to a few strains of *Escherichia*.

The *Salmonella* strains were frequently sensitive to *Escherichia* (22 per cent of the trials showed growth inhibition), occasionally sensitive to *Citrobacter* and paracolon strains, but never sensitive to the antibiotic *Shigella* or *Eberthella* strains employed.

In table 3 is shown the frequency of growth inhibition by and among enteric bacteria. Of 56 strains of *Escherichia* that were tested for sensitivity to various other enteric forms, a large proportion (25 to over 75 per cent) were sensitive to a large number of other *Escherichia* strains, whereas only a few were sensitive to *Shigella* or *Eberthella* strains.

Among 14 *Aerobacter* strains tested for sensitivity against other members of the *Enterobacteriaceae*, only 3 (or less than 25 per cent) were inhibited by *Shigella* and that was due to a single *Shigella* strain; 25 to 49 per cent of the *Aerobacter* showed some evidence of sensitivity to *Escherichia* strains, but there again only a small proportion of *Escherichia* strains (8 per cent) were antibiotic.

It will be seen from table 3 that the 33 strains of *Salmonella* tested were never sensitive to any of the strains of *Shigella* or *Eberthella* and only occasionally sensitive to some *Citrobacter* and paracolon bacilli; but *Escherichia* strains were frequently antibiotic against *Salmonella* strains, as indicated by 41 per cent of the 39 *Escherichia* cultures employed showing antibiotic action against 25 to 75 per cent of the *Salmonella* strains under observation.

Considering the *Shigella* strains, it again will be noted that these were particularly susceptible to *Escherichia* strains (95 per cent of the 39 antibiotic *Escherichia* strains being effective against 25 per cent or more of the *Shigella* cultures). They were also sensitive to strains of *Citrobacter* and to paracolon bacilli, but particularly to other *Shigella* strains (47 per cent of them being active against more than 50 per cent of the *Shigella* cultures tested for sensitivity).

The insensitivity of *Proteus* strains is evident as only 2 (5 per cent) of the 39 *Escherichia* cultures were antibiotic against them, but these *Escherichia* strains were effective against 6 of the 8 strains of *Proteus* observed.

In table 4 are shown the results obtained with one strain of *Escherichia* (37) and one strain of *Shigella sonnei* (P9) against each of 193 enteric bacteria. Considering the *Escherichia* strain, it will be noted that it was antibiotic against 85

TABLE 3  
Frequency of growth inhibition by and among enteric bacteria

ANTIBIOTIC ENTERIC STRAINS....	<i>Escher- ichia</i> 39	Para- colon 21	<i>Citro- bacter</i> 8	<i>Eber- thella</i> 5	<i>Shig- ella</i> 15	ANTIBIOTIC ENTERIC STRAINS....	<i>Escher- ichia</i> 39	Para- colon 21	<i>Citro- bacter</i> 8	<i>Eber- thella</i> 5	<i>Shig- ella</i> 15
Per cent of 56 <i>Escherichia</i> inhibited	Per cent of various strains antibiotic against <i>Escherichia</i>					Per cent of 13 <i>Citrobacter</i> inhibited	Per cent of various strains antibiotic against <i>Citrobacter</i>				
0						0	72	76	12		93
<25	31	81	50	100	100	<25	18	14	88	100	7
25-49+	54	14	50			25-49+		5			
50-74+	10	5				50-74+	2	5			
75+	5					75+	8				
Per cent of 23 paracolon inhibited	Per cent of various strains antibiotic against paracolon					Per cent of 14 <i>Aerobacter</i> inhibited	Per cent of various strains antibiotic against <i>Aerobacter</i>				
0	13				33	0	92	100	100	100	93
<25	69	90	75	100	67	<25					7
25-49+	10	10	25			25-49+	8				
50-74+	8					50-74+					
75+						75+					
Per cent of 33 <i>Salmonella</i> inhibited	Per cent of various strains antibiotic against <i>Salmonella</i>					Per cent of 41 <i>Shigella</i> inhibited	Per cent of various strains antibiotic against <i>Shigella</i>				
0	26	67	75	100	100	0	2				7
<25	33	24				<25	3	14	12	100	40
25-49+	28	9	25			25-49+	77	81	50		6
50-74+	13					50-74+	8	5	38		47
75+						75+	10				
Per cent of 8 <i>Proteus</i> inhibited	Per cent of various strains antibiotic against <i>Proteus</i>					Per cent of 6 <i>Eberthella</i> inhibited	Per cent of various strains antibiotic against <i>Eberthella</i>				
0	95	100	100	100	100	0	36	67			80
<25						<25	54	23	75	100	20
25-49+						25-49+		5	25		
50-74+						50-74+		5			
75+	5					75+	10				

per cent of the 56 *Escherichia*, 70 per cent of 23 paracolon bacilli, 85 per cent of 13 *Citrobacter* strains, 100 per cent of 6 *Eberthella* strains, and 80 per cent of 41 *Shigella* strains, compared with only 27 per cent of the 33 *Salmonella* strains. It was entirely inactive against the 7 *Proteus* and the 14 *Aerobacter* strains that were observed.

The growth inhibition zones varied markedly for different strains, indicating a large variation in the degree of resistance or susceptibility of the various strains tested to the antibiotics produced by this *Escherichia* strain (37). Perhaps a particularly striking observation is that among the 33 strains of *Salmonella* tested for sensitivity to this strain of *Escherichia*, 24 (73 per cent) were insensitive; but for the 9 sensitive strains the diameter of the inhibition zone was over 40 mm in eight instances (in three of which it was over 50 mm in diameter). The 9 highly sensitive strains of the *Salmonella* included an unidentified strain,

TABLE 4  
*Growth inhibition zone against various enteric bacteria*

DIAMETER INHIBITION ZONE		NONE	<10 MM	10-19 MM	20-29 MM	30-39 MM	40-49 MM	50-59 MM	PER CENT STRAINS INHIBITED†
Test strains		Number and per cent* of test strains inhibited by <i>Escherichia</i> strain 37							
Group	No.								
<i>Escherichia</i>	56	7 (13)	1 (2)	10 (18)	7 (12)	26 (46)	5 (9)		85
<i>Paracolon</i>	23	6 (26)	1 (4)	4 (17)	4 (17)	2 (9)	6 (26)		70
<i>Citrobacter</i>	13	2 (15)		2 (15)	3 (23)	5 (39)	1 (8)		85
<i>Aerobacter</i>	14	14 (100)							
<i>Proteus</i>	7	7 (100)							
<i>Salmonella</i>	33	24 (73)				1 (3)	5 (15)	3 (9)	27
<i>Eberthella</i>	6			5 (83)		1 (17)			100
<i>Shigella</i>	41	8 (19)	2 (5)	13 (32)	12 (29)	5 (12)	1 (2)		80
		Number and per cent* of test strains inhibited by <i>Shigella sonnei</i> strain P 9							
<i>Escherichia</i>	56	42 (75)	2 (4)	5 (9)	4 (7)	3 (5)			21
<i>Paracolon</i>	23	19 (83)	1 (4)	2 (9)	1 (4)				13
<i>Citrobacter</i>	13	11 (84)		1 (8)	1 (8)				16
<i>Aerobacter</i>	14	13 (93)		1 (7)					7
<i>Proteus</i>	7	7 (100)							
<i>Salmonella</i>	33	33 (100)							
<i>Eberthella</i>	6	5 (83)	1 (17)						
<i>Shigella</i>	41	12 (29)		5 (12)	22 (54)	2 (5)			71

\* Per cent to nearest whole number.

† Showing inhibition zones of at least 10 mm.

the only *S. tennessee* and *S. newport*, each of the two *S. enteritidis*, and the four *S. schottmuelleri* of our collection. It would be interesting, and perhaps significant, to determine whether other strains of these *Salmonella* types are similarly sensitive to *Escherichia* strain no. 37.

The inhibition zones produced by the *Shigella sonnei* strain (P9) were very much smaller than those frequently observed with the *Escherichia* strain referred to above, and it will be noted that it was effective particularly against other strains of *Shigella*; 29 (or 71 per cent) of the 41 *Shigella* strains tested for sensitivity showed inhibition zones.

As illustrated by these two examples, the antibiotic spectra of various active

strains appear to be specifically characteristic. The sensitivity of a particular strain toward these antibiotics may differ widely even among members of the same group. However, when a strain of a well-defined type of *Salmonella* or *Shigella*, such as *S. schottmuelleri* or *Shigella sonnei*, was found to be sensitive to one or more of the active cultures, all other strains of the same type available at the time of this study were likewise susceptible to the antibiotics produced by those particular active cultures. This indicates that there may be some correlation between biochemical properties, or antigenic structure, and antibiotic sensitivity.

#### SUMMARY

The results obtained with 88 antibiotic enteric strains against 194 members of the *Enterobacteriaceae* (including the antibiotic strains), employing a simple technique for rapidly ascertaining sensitivity or antibiotic activity, are described.

Members of the genus *Shigella* were more frequently sensitive to antibiotics produced by various enteric strains than were other genera of the *Enterobacteriaceae*.

The frequency of sensitivity to antibiotics produced by various members of the *Enterobacteriaceae* decreased in the following order: *Shigella*, *Escherichia*, paracolon bacilli, *Citrobacter*, *Salmonella*, *Proteus*, and *Aerobacter*. Thus, 40 per cent of 3,593 trials employing strains of *Shigella*, 28 per cent of 4,928 trials with *Escherichia*, 15 per cent of 2,024 trials with paracolon bacilli, 12 per cent of 2,904 trials with *Salmonella*, 2 per cent of 704 trials with *Proteus*, and 1 per cent of 1,232 trials with members of the genus *Aerobacter* showed evidence of sensitivity to antibiotics produced by members of the *Enterobacteriaceae*.

*Shigella* strains were frequently active against *Escherichia*, particularly active against other *Shigella* strains, only occasionally active against *Citrobacter*, and entirely inactive against members of the genera *Aerobacter*, *Proteus*, or *Salmonella*.

*Escherichia* strains, on the other hand, are not only active against many other strains of *Escherichia*, *Citrobacter*, paracolon bacilli, and *Shigella*, but also against many *Salmonella*, though only occasionally against strains of *Aerobacter* or *Proteus*.

When a well-defined type of *Salmonella* or *Shigella*, such as *Salmonella schottmuelleri* or *Shigella sonnei*, was found to be sensitive to one or more active cultures, all available strains of the same type were similarly sensitive to those active cultures, indicating that there may be some correlation between biochemical properties, or antigenic structure, and antibiotic sensitivity.

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## NOTE

### BACILLIN PRODUCTION BY SOIL ISOLATES

F. J. RUDERT AND MILTON J. FOTER

*Department of Bacteriology, Research Laboratories, The Wm. S. Merrell Co., Cincinnati, Ohio*

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A series of 46 soil samples were examined for bacilli antagonistic to *Escherichia coli*. Pasteurized soil suspensions were diluted in an agar medium containing 0.5 per cent of yeast extract, tryptone, and glucose. After 2 days at 26 C, the plates with limited numbers of discrete colonies were sprayed with a tenfold dilution of an *E. coli* culture, using an ordinary atomizer. The following day the colonies surrounded by clear zones of inhibition were picked and purified in the usual manner. Twenty-three soil samples from 15 states yielded 46 isolates producing relatively large zones and exhibiting similar colonial characteristics. The colonies were very viscous and wrinkled and exhibited a tendency to run on inclination of the plate.

The active material was relatively heat-stable but was totally inactivated by hydrogen sulfide. The antibacterial activity was greatly reduced in complex media such as brain-heart infusion. It was not extractable with organic solvents but could be adsorbed on various activated carbons and partially eluted with aqueous ethanol. The wide antibacterial spectrum and the foregoing properties indicate an identity with bacillin (Foster and Woodruff; J. Bact., 51, 363).

Adsorption on the cation resin, "ionac C284" (American Cyanamide and Chemical Corporation), followed by elution with dilute aqueous pyridine gave a 50-fold increase in potency and was the most satisfactory procedure for preliminary purification. Further measures were ineffectual, and lyophilization of concentrates usually resulted in a marked loss in activity.

The widespread occurrence of this organism and the marked antagonistic properties in the absence of complex nutrients suggest that it may have a role in controlling the microbial flora of the soil.





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